THE ANTIBACTERIAL EFFECTS OF RADIOPAQUE DOUBLE ANTIBIOTIC PASTES AGAINST CLINICAL BACTERIAL ISOLATES FROM MATURE AND IMMATURE TEETH WITH NECROTIC PULPS

by

Carolin Francis Ibrahim

Submitted to the Graduate Faculty of the School of Dentistry
in partial fulfillment of the requirements for the degree of
Master of Science in Dentistry, Indiana University
School of Dentistry, 2018.

Thesis accepted by the faculty of the Department of Endodontics, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

_____

Ghaeth Yassen

_____

Ygal Ehrlich

_____

Richard L. Gregory

_____

Susan Zunt

_____

Josef Bringas

_____

Kenneth J. Spolnik
Endodontic Chair and
Graduate Program Director

_____

Date

ACKNOWLEDGMENTS

First and foremost, I would like to thank my parents, Bernadet and Francis, for teaching me what love, support, and perseverance are in the face of the biggest challenges in life. All that I am and do, I owe it all to you. To my father, who left this earth 20 years ago – my 10 years with you taught me more than I could learn on my own in a lifetime. I hope I have made you proud.

Thank you to my dear husband and my biggest champion, Chris. You have been unconditional in all ways since the day we met. Your unwavering patience, love, and selflessness have allowed me the chance to pursue my dreams. It has been a long road, and you have always remained my biggest supporter. I could not have done any of this without you.

To Dr. Spolnik – my deepest gratitude goes to you for believing in me and providing me with a life-changing opportunity. You are a beacon of knowledge and I thank you for imparting your expertise to make us the best clinicians in the country. There truly is no greater program director. On a day-to-day basis, you taught us about endodontics and business and even more importantly about life. I am blessed to have trained under your leadership.

I owe a great thank you to the person who spearheaded this study and whose guidance was so instrumental to its completion, Dr. Yassen. Thank you for your hard work and supervision, and for spending countless hours answering my questions. You saw this project to the end even after moving away, and I thank you for your endless support.

Dr. Bringas, I have been so fortunate to train under you not only in Indianapolis, but in Detroit as well. Thank you for challenging us to be independent thinkers and doers. You never mandated one way of thinking on us. Rather, you always challenged us to look at the evidence and make decisions for ourselves. You have groomed us to excel and to always do our best, and I thank you for being one of my most pivotal mentors.

Thank you to Dr. Ehrlich for using your prolific knowledge of research to help me to appreciate the same kind of passion as yours for endodontic literature. Your dedication to research is invaluable, and it has allowed us as residents to excel not just as clinicians, but also as contributors to endodontic research.

Dr. Warner, thank you for having such a great attitude during our time in residency. Your positivity taught me to be patient, especially while instructing the pre-doctorate students.

Thank you, Dr. Gregory, for your unending willingness to improve this thesis project. Thank you for challenging and motivating me to study hard and to learn so much in your class and lab.

To all of our staff and assistants, Jenny, Linda, Karen, Steve, Mary, and Indu – I am grateful to have worked so closely with you. Thank you for your kindness, hard work, and patience. I have learned as much about endodontics from all of you as I have from our faculty, and I would not have matured as a clinician without all your help.

Many thanks to all part-time faculty who sacrifice their time week after week for our benefit as residents. Drs. Vail, Adams, Hill, Steffel, Berman, Sahni, Deardorf, Prather, and Hine, you each brought invaluable individual strengths to the Graduate Endodontics program that have helped shape me clinically and professionally. You are

some of the most knowledgeable, talented, and supportive clinicians in the country. I hope to carry on your strive for excellence into the real world.

Ross, Kathryn, and all my co-residents – Thank you for welcoming me into your lives and forever enriching mine. The companionship and laughs we shared in the endodontic conference room on a daily basis is second to none, and I thank each and every one of you for your sincere friendship. You pushed me to be better day by day. I could not have asked for better co-residents. Thank you sincerely.

I will always be grateful to everyone in the Graduate Endodontics department for propelling me in my professional and personal life. You are an extension of my family, and because of you my experience in residency will forever be cherished.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

INTRODUCTION

During the stages of tooth development, trauma or introduction of bacteria into the pulp can lead to inflammatory changes with eventual pulpal necrosis.[1]. Consequently, this presents a challenge in clinical situations; the root development is interrupted resulting in thin dentinal walls and wide-open apexes, which even when treated results in a marked decrease in fracture strength.[2-4]. These teeth are difficult to treat with conventional root canal therapy due to struggles with instrumentation and disinfection.[5] Additionally, obturation is challenging because of a lack of an apical barrier to contain the obturation material within the canal system.[5]

In the past few decades, treatment modalities for immature teeth with necrotic pulps have evolved. Apexification has been frequently used to treat incomplete apical closure in teeth with pulpal necrosis. In this procedure, calcium hydroxide is placed long-term in the canal system to form a calcified barrier across the apex.[6-8] Obturation of the canal system is then made possible by the formation of this barrier against which gutta percha can be condensed minimizing the fear of overextension of the material into the periradicular tissues.[5,9,10]. More recent endodontic advancements have also introduced Mineral Trioxide Aggregate (MTA), which has a consistency compared to calcium hydroxide, as a treatment modality in the formation of an apical barrier.[11] The greatest limitation in apexification with calcium hydroxide or MTA is that these techniques do not increase the root length or thickness in immature teeth with pulpal necrosis, and this presents many clinical problems.[12] As a result, these teeth have an elevated risk for fracture.[2-4, 13]

Fortunately, regenerative endodontic procedures (REPs) have emerged as an alternative to the aforementioned apexification techniques. REPs utilize the concept of tissue engineering, and the three requirements needed for this are stem cells, scaffolds, and growth factors.[14] The current guidelines for regenerative endodontics as recommended by the American Association of Endodontists (AAE) state the need for disinfection and initiation of bleeding into the canal to produce a blood clot.[15] The induction of bleeding evokes an influx of multipotent mesenchymal stem cells from the apical papilla into the canal system.[16] The accumulation of this blood forms a clot, which serves as a fibrin scaffold for the stem cells to proliferate and differentiate.[16-18] Proliferation and differentiation is achieved with the aid of growth factors, which are released from the dentin and platelets within the canal space.[18,19] Several research trials have reported successful elimination of disease as well as an increase in root length and width using these REP protocols.[12, 20]

Disinfection is an essential component of regenerative endodontic procedures. Minimal to no mechanical debridement is performed in REPs in comparison to conventional root canal therapy.[21, 22] Chemical debridement and intracanal medication are used in REPs as a means of achieving disinfection and the resolution of infection.[21,22] Some of the most common agents recommended in the irrigation and disinfection phase of REPs are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), calcium hydroxide ($Ca(OH)_2$), triple antibiotic paste (TAP), and double antibiotic paste (DAP).[21]

NaOCl has been widely used as the primary root canal irrigant in concentrations of 0.5%-5.25%.[23] It has a significant antimicrobial activity along with the ability to

dissolve organic tissues.[23] However, NaOCl is used in lower concentration (1.5%) in REPs due to its cytotoxic effect on stem cells in higher concentrations.[24] When 17% EDTA is used with NaOCl, its detrimental effects on the stem cells are reversed.[18] EDTA is a chelating agent that removes the inorganic portion of the dentinal smear layer thus exposing dentinal tubules and collagen fibrils as well as enabling the release of growth factors from the dentin matrix.[19, 25, 26] As stem cells are introduced into the canal they can form an intimate association with the EDTA treated dentin. [18] These stem cells may differentiate into odontoblast-like cells with processes that extend into the dentinal tubules.[18]

Calcium hydroxide has been commonly used as an intra-canal medicament in regenerative endodontics. $Ca(OH)_2$ is a bactericide with an alkaline pH of 10-12 that has been shown to inactivate and detoxify lipopolysaccharide (LPS) endotoxins.[27-30] However, its long-term use can have negative effects on the mechanical properties of radicular dentin including a reduction in fracture strength[2, 3] and superficial collagen and dentinal protein degradation [31] resulting in dentinal weakening.

Several antibiotic pastes with variations in types and concentrations have been utilized as intra-canal medicaments in regenerative endodontics. The most common of these is a triple antibiotic paste (TAP) composed of ciprofloxacin, metronidazole, and minocycline, which has been shown to be effective in disinfecting the canal system.[15, 32, 33] However, TAP presents with several negative effects including dentin demineralization, tooth discoloration, and cytotoxicity of stem cells in higher concentrations.[31, 34, 35] DAP, made of metronidazole and ciprofloxacin, has also been used successfully as a disinfectant in REPs. [36] Minocycline has been eliminated in this

formulation in order to avoid its staining characteristics.[5] However, despite its proven efficacy, DAP remains cytotoxic to stem cells in higher concentrations.[37]

Some studies have utilized DAP as an intracanal medicament and demonstrated its success in regenerative endodontics. In a pioneering case report, a double antibiotic combination of ciprofloxacin and metronidazole was used to treat an immature mandibular premolar with a necrotic pulp.[36] The start of apical closure was demonstrated 5 months after completion of the treatment protocol.[36] After 30 months post-treatment, complete apical closure and root lengthening and thickening was observed hinting at the potential for revascularization in a young permanent tooth.[36] Nevins and Cymerman have supported this finding in a recent case series report. Apart from root development and lengthening, their case series reported a reduction in symptoms and periapical radiolucency size with the use of DAP.[38] They also concluded that tissue repair was not adversely affected with the elimination of minocycline from the antibiotic paste regimen.[38]

The effectiveness of DAP against common endodontic pathogens such as *E. faecalis* and *P. gingivalis* has been compared to TAP and calcium hydroxide in clinical studies. In a study by Sabrah et al, it was found that both DAP and TAP performed equally in their antimicrobial efficacy, which was superior to calcium hydroxide. [39] An added advantage with DAP is that it does not discolor teeth and the authors concluded that it can be considered an effective substitute to TAP for that reason.[39] Another advantage of DAP is its lack of negative effects on dental pulp stem cells (DPSCs) when used in lower concentrations.[40, 41] In one study, two dilutions of DAP (500 and 1 mg/mL) were tested with and without EDTA rinse to assess the proliferation and

attachment of DPSCs. [41] It was found that both concentrations of DAP with the use of EDTA irrigation significantly enhanced the DPSCs attachment to dentin.[41] The lower concentration of 1 mg/mL DAP was also found to lack any significant negative effects on DPSCs proliferation.[41] Implementing DAP in REPs could be advantageous for these reasons.

A number of studies have supported the current concept that several bacterial species are implicated in the etiology of endodontic infections.[42-44] These studies confirm the presence of multiple clinical isolates that can be found in bacterial biofilm in endodontic infections. For this reason, it is important to replicate the antimicrobial effects of intra-canal medicaments against clinical bacterial biofilms and to determine if there is a difference between those obtained form a mature tooth compared to an immature tooth.

Ideally, the intracanal medication used in REPs should have radiopaque properties to radiographically confirm its placement in the root canal.[45,46] Commercially available calcium hydroxide medicaments contain 30-40% of barium sulfate ($BaSO_4$) as a radiocontrast agent to confirm that the calcium hydroxide has been placed sufficiently into the canal system. The radiocontrast agents commonly used in dental materials are insoluble salts of heavy metals such as barium, zirconium and bismuth. Currently antibiotic combinations used in REPs do not contain any radiopaque materials. Therefore, over or under application of non-radiopaque antibiotic medicaments can easily go unnoticed during REPs because of the open immature apices of teeth with necrotic pulps. Barium sulfate has been suggested as a radiopaque agent in various endodontic materials including medicaments, bioceramics, and gutta percha. A recent project conducted at

IUSD has suggested the use of radiopaque DAP with 30% barium sulfate as radiocontrast agent. The suggested radiopaque medicament was found to have comparable radiodensity to Ultracal, which is the most commonly used commercial calcium hydroxide medicament (Figure 24). Furthermore, a recent pilot study conducted at IUSD found that both radiopaque and non-radiopaque 1 mg/mL DAP had significant antibacterial effect against a 3 week old bacterial biofilm (Table I). For this reason, it is our focus to investigate radiopaque DAP (RoDAP) and evaluate its antimicrobial effect against clinical bacterial isolates from mature and immature teeth with necrotic pulps.

OBJECTIVE

The objective of this experiment is to investigate the antibacterial effect of 1 mg/mL and 10 mg/mL radiopaque DAP on radicular dentin infected with clinical bacterial isolates from mature and immature teeth with necrotic pulps.

NULL HYPOTHESES

The null hypotheses are as follows:

- The 1 mg/mL and 10 mg/mL concentrations of radiopaque DAP will not have significant antibacterial effect against bacterial isolates obtained from mature and immature teeth with necrotic pulps.
- The tested concentrations of radiopaque DAP will demonstrate similar antibacterial effects against bacterial isolates obtained from immature and mature teeth with necrotic pulps.

ALTERNATIVE HYPOTHESES

The alternative hypotheses are as follows:

- The 1 and 10 mg/mL of radiopaque DAP will have significant antibacterial effects, regardless of the source of bacterial isolates.
- The tested concentrations of radiopaque DAP will demonstrate significantly lower antibacterial effect against bacterial isolates obtained from an immature tooth with necrotic pulp in comparison to that obtained from a mature tooth with necrotic pulp.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Dentistry's history is fairly rich and the treatment of dental pain is deeply rooted in several ancient practices and cultures. The etiology of dental disease can be traced back several thousand years ago where it was believed that a "tooth worm" caused caries, toothaches, and periodontitis. Several accounts of this can be seen dating back to Sumerian texts around 5,000 BC. [47] Other interpretations of the dental tooth worm can be found in different cultures before this belief was popularized in western cultures as the cause of dental disease.

It was not until the 18th century that the theory that a tooth worm causes dental caries and periodontitis began being refuted by discoveries made Anton Von Leeuwenhoek who observed microorganisms in tooth samples. A shift in the dental disease paradigm came when Pierre Fauchard, known as the father of modern dentistry, dispelled the theory of the dental tooth worm and theorized that decay was linked to sugar consumption. Further discoveries made in the 1890s by Willoughby Dayton Miller postulated that caries is caused by oral bacteria producing acid after the consumption of fermentable sugars.[48]

Many of today's concepts of dentistry and endodontics can be attributed to a shift in the practice of extracting and replacing teeth to that of treating oral disease and maintaining the dentition. In the 1700s, treatment of pulpal disease involved using various chemicals and medicaments and obturating the pulp chamber only. In 1728, however, Pierre Fauchard described, in his book *Le Chirurgien Dentiste,* several dental

procedures that have been refined into modern concepts and practices in endodontics. Fauchard described one such procedure that highlighted how a dental abscess was drained by creating an access into the pulp space, which was left open for days to several months. Thereafter, following adequate drainage, Fauchard described sealing the access and canal space using lead foil making this rudimentary procedure one of the first of its kind to obturate canal spaces.[49, 50] In his book Fauchard also detailed extirpating dental pulps using instruments such as small pins as well as the application of cinnamon and cloves to extensive caries which were basic modes of treatment that paved the way for modern techniques of pulp extirpation and pulp capping.[51, 52]

Other dental innovators in the 18th century, such as Philip Pfaff and W.W. Codman, further elaborated on these endodontic pulp capping procedures. Pfaff, twenty eight years later, attempted the maintenance of exposed dental pulps by covering the exposure with concave pieces of lead or gold made to fit the opening.[49] Furthermore, in 1850, W.W. Codman confirmed that the objective of pulp capping was to form secondary dentin and form a dentin bridge after pulp exposure.[53]

In the middle of the 18th century, Robert Woofendale was successful in completing the first endodontic procedure. In order to alleviate pain, he implemented the use of a heated instrument to cauterize the pulp and placed cotton pellets into the canals thereafter.[53, 54] He also advocated the use of oil of cinnamon, cloves, turpentine, opium, and camphor in the alleviation of odontogenic pain.[53] Further advancement in diagnosis was elucidated when Frederick Hirsch began evaluating the apical tissues through percussion tests. His tests discovered that pain upon tapping was associated more with

diseased teeth, and similarly to Woofendale's methods, he recommended the use of a hot probe to cauterize the pulp and alleviate pain.[52]

Beginning in the 19th century, "The Vitalistic Era" emerged. This era marked a change in opinion and practice whereby the dental community recognized the importance of a vital pulp and the subsequent effects of its treatment.[49] At this time, pulpal circulation was explained by Charles Bew; he detailed the flow of blood entering into the pulp through the apical foramen and then exiting through the periodontal membrane.[55] In 1826 Leonard Koecker, in his book titled *Principles of Dental Surgery,* postulated that removing the pulp would result in further necrosis of the remaining tissues and would render the tooth a foreign body. [55] In order to avoid a foreign body reaction after removal of pulp tissues, Koecker advocated the implementation of pulp capping procedures similar to those proposed by Pfaff.[53, 55].

In 1829, S.S. Fitch published *System of Dental Surgery,* where he detailed his vitalistic theory which stated that the tooth in its entirety was vital, similar to bones. In addition, he stated that pulpal blood supply was essential to and supplied the coronal tooth and that pulp and periodontal ligament both supplied the root of the tooth. Decoronation began to be practiced whereby the crown of the tooth was removed after the pulp and blood supply had been extirpated and the root was left in its socket for further restoration. Conversely, those supporting a nonvitalistic school of thought, postulated that enamel and dentin are devoid of circulation and therefore lack in abilities such as sensibility and the capacity to heal. This theory promoted the idea that removal of pulpal tissues and blood supply would not affect the integrity of the remaining tooth structure.[55]

The following decades saw the introduction of several new medicaments used in endodontics which were used to devitalize the pulp and alleviate the pain associated with pulp extirpation. One of the first to do so was Shearjashub Spooner in 1836, who implemented a technique used in ancient Chinese medicine that used arsenic trioxide to devitalize the pulp prior to pulpectomy.[56] This became popular and remained in practice until the 1920s due to its success in relieving pain during vital pulpectomies.[57] In the 1940s, formocresol was introduced by John P. Buckley for pulp fixation which continues to be used to some extent in modern practice.[58] Meanwhile, other practitioners such as Jacob and Joseph Linderer promoted using essential or narcotic oils over pulp exposures for pain alleviation.[59]

The 19th century saw the introduction of new practices for root canal filling and sealing. One of the pioneers of this, Edward Hudson, in 1809, developed instrumentation for packing root canals with gold foil.[56] This was further developed by Baker who has been credited with the first description of the core ideas of pulp extirpation, root canal cleaning, and filling of the canal space. In the 1839 American Journal of Dental Science, Baker described removal of nerve tissue and debridement of the canal in addition to obturation with gold foil.[49] Later, in the 1850s, another method of obturation was advocated which utilized plugs of beechwood saturated with creosote.[50].

It was shortly thereafter that gutta percha, the contemporary root-filling of choice, was introduced by Dr. G. A. Bowman.[50] Clarke Dubuque furthered this process by introducing heated gutta percha.[49] By the end of the 19th century Dr. Bowman advanced the use of gutta percha by developing a new technique which gained much popularity for several decades. This was the introduction of chloropercha, which used chloroform to

soften the gutta percha and allow for better adaptation of the obturation material in the canal system.[56].

Modern endodontic armamentarium including basic instruments such as files and the rubber dam were invented in the 1800s. The development of the first instrument used to extirpate pulp tissue is credited to Edwin Maynard; he developed what would resemble a modern broach by winding a watch spring. In Monticello, NY, Sanford Barnum invented the rubber dam in 1862 and began using it for isolation during preparation and placement of gold foil restoration.[50] The rubber dam would later become a huge staple and part of standard of care in modern endodontics due to its ability to provide an aseptic environment, free of saliva, during endodontic therapy.

With the endodontic community understanding the role of microorganisms in pulpal and periapical disease, establishing an aseptic working field would become vital to the practice of endodontics. In his 1878 *Dental Cosmos* article, Dr. G. O. Rodgers commented that pathogenic microorganisms might be an insult to the pulp resulting in pulpal pathoses. Thus, complete eradication of this microbial insult would remedy the disease and provide successful treatment.[51] In 1882, Arthur Underwood expanded on this by suggesting that the use of antiseptics could eliminate microbes and treat pulpal suppuration.[49]

The turn of the 20th century brought on groundbreaking advances in dentistry and endodontics with the introduction of revolutionary technology. These included the introduction of local anesthetics and dental radiology. Of these, the development of procaine (Novocaine) in 1905 would become a hallmark of dentistry, replacing cocaine, the anesthetic agent used previously. Combined with block anesthesia, which was

developed in the 1920s, this resulted in far more superior efficacy of local anesthetia.[57, 60] Another monumental technological advancement at the time was the advent of the x-ray unit to dentistry and endodontics. After its introduction, the dental x-ray unit was not commercialized until after 1919 with the introduction of the Coolidge tube which allowed for a more focused and precise x-ray beam.[51] As a result, this revolutionized our understanding and treatment of endodontic disease as periapical pathosis could now be visualized radiographically; a connection could be made between pulpal and periapical disease.[61] Radiography allowed for more detailed and precise work in endodontics, because it could be implemented in root length determination during cleaning and obturation procedures. By implementing this new technology, our understanding of root canal anatomy would later be amplified and become more precise and comprehensive.[58]

At the time of these dental advances, the practice of endodontics was scrutinized by the introduction of the "Focal Theory of Infection." This theory suggested that dental infections could disseminate pathogenic microorganisms and their toxins into systemic circulation and cause various diseases.[62] A British physician and pathologist, William Hunter of McGill University, especially popularized this theory in 1911 when he published a lecture titled "The Role of Sepsis and Antisepsis in Medicine." [62,63] Hunter stated that "gold fillings, gold caps, gold bridges, gold crowns, fixed dentures, built in, on, and around diseased teeth, form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine or surgery."[64] In the wake of this ideology, physicians began recommending extraction of many teeth that were endodontically treated, non-vital teeth, or even teeth that were restorable but compromised. Some physicians advocated even more severe recommendations such as

prophylactic removal of all teeth to prevent or treat systemic disease.[62] The decades that followed, referred to as "the scientific era" by some, were a time of advances in the medical and dental community that undermined the focal infection theory. This theory was finally discredited in the 1930s and 1940s when a clear causation of systemic disease could not be linked to dental infections.[63]

The 1920s and 1930s began the use of an advancement that redefined endodontic medicaments, calcium hydroxide. Hermann first started using this in vital therapy procedures as well as an intracanal medicament. He believed that endodontic materials spread through the tooth and became absorbed by the surrounding oral tissues; therefore, he advocated the use of more biocompatible alternatives in place of previously used caustic medicaments. He was able to demonstrate that a dentin bridge could be formed with the use of calcium hydroxide.[63]

The first obturation cement was developed by U.G. Rickert which would be a way to improve upon the obturation of the canal system. In his technique, Rickert coated the gutta percha cone with sealer before inserting it into the canal. Further techniques to improve obturation were implemented by the introduction of instruments to laterally condense gutta percha. At this same time, lentulo spirals were introduced and could be used to deliver sealer as well as calcium hydroxide into the canals.[63]

Dentists began using antibiotics for dental infections following the advent of the first antibiotic, penicillin. Adams and Grossman first introduced antibiotics as an adjunct to endodontic therapy in the 1940s.[63] Grossman suggested using penicillin with a more localized delivery into infected canal systems and recommended applying it in a non-aqueous preparation. Later, in order to sterilize the canal, he advocated using paper points

impregnated with penicillin.[56] However, it was realized that penicillin alone could not sterilize the canal system. Thus began an interest in the chemotherapeutic management of endodontic infections alongside the physical debridement. The combination of chemotherapeutic agents along with mechanical preparation gave rise to the idea of chemo-mechanical preparation, which would later form the foundation in cleaning and shaping in modern endodontics.[65]

With the foundation of the American Association of Endodontists (AAE) in Chicago, Illinois in 1943, organized endodontics was finally established and the profession could be directed under one body. In 1949, the AAE pushed the idea of creating a specialty board, and thus the American Board of Endodontics (ABE) was founded in 1956 [66]. However, it was not until 1963 that American Dental Association (ADA) finally recognized endodontics as a specialty due to the dedicated hard work and strong leadership of its members.[63]

The late 1900s and the turn of the 21st century introduced many monumental advances in endodontics. To name a few, nickel titanium rotary files, the dental operating microscope with its advanced illumination and magnification, cone-beam computed tomography, and bioceramic material such as mineral trioxide aggregate (MTA) have propelled the current practice of endodontics.[67-72] More recently, regenerative endodontics has emerged as a popular treatment modality, and just over a decade ago the first regenerative endodontics conference took place at Nova Southeastern University.[73] Just a few years ago, in 2012, the ADA added a new code for pulpal regeneration cementing the idea that this treatment option is quite viable. Despite all the

ongoing research and advances in regenerative endodontics, there is still much knowledge to be gained about this treatment modality.

THEORY OF ENDODONTICS

As we now know bacterial insult can be instrumental in initializing endodontic disease through the introduction of pathogens into the pulpal tissues by caries or trauma. One of the most influential studies, which helped shape and support this idea, was that of Kakehashi, Stanley, and Fitzgerald in 1965. Their study was able to demonstrate that exposed pulps in germ-free rats remained vital despite incurring trauma from mastication and exposure to the oral environment. However, under the same conditions, rats that were not germ-free demonstrated necrosis of the pulp and periapical pathosis.[74] As a result, the conclusion reached was that bacteria are responsible for pulpal and periapical disease. The deductions garnered from this study continue to impact our practice of endodontics today.

As a result of the knowledge gained from studies like those of Kakehashi et al., we have now established that achieving maximal reduction in pathogens and their toxins is one of our primary goals in endodontics. [75,76] This goal is achieved by debriding the canal system mechanically through physical cleaning and shaping and chemically via the application of irrigants and intracanal medicaments.[75, 76] Periapical and periradicular inflammation can be an extension of pulpal disease within the canal system as endodontic pathogens and their byproducts exit to the periodontium through the apex and lateral or accessory canals.[77] In the case that endodontic therapy is unable to eradicate and minimize the number of these microorganisms within the canal system, the eventual result seen is inflammation of the pericapical tissues known as apical periodontitis.[78]

Success of endodontic treatment is, therefore, directly related to the reduction of pathogenic bacteria.[74,79]

In the middle of the 20th century, Dr. G.G. Stewart highlighted the three main phases of treatment faced in endodontics: chemomechanical preparation, control of microbes, and sealing and obturation of the root canal system.[80] The most crucial of these, chemomechanical preparation reduces the microbial load while creating a space for obturation. Later, Grossman was able to expand on Stewart's theory and corroborate his finding by confirming that the chemomechanical process eliminates bacteria and debris from root canals.[81] He was instrumental in identifying 13 principles to be followed during any root canal procedure:

1. Use of aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irrigating to the periapical tissues.
11. If an acute alveolar abscess is present, proper drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

The fundamental objective of endodontic therapy, as it is known today and was discussed by Schilder in 1967, is the removal of the necrotic tissues and the canal contents that are responsible for inflammation of periapical tissues. Schilder believed not only in effective chemomechanical debridement, but also stressed the importance of adequate obturation of the canal system in three dimensions. The obturation technique he introduced in 1967 involved filling the canal in "three dimensions" whereby small increments of gutta percha placed into the canal were heated with a spreader and a plugger was used to condense the filling thereafter; this allowed for a more dense and homogenous root canal fill.[82]

Pitt Ford resonated with Schilder's technique for obturation in three dimensions and cited three concepts for supporting this type of fill.[83] First, he stated that it allowed less space for bacterial colonization. Second, apical contamination could be prevented with a three-dimensional fill. Lastly, movement of bacteria along the periphery of the canal could be prevented.[83] Additionally, Pitt Ford stressed the necessity of an aseptic endodontic technique to prevent further contamination of the root canal system. He listed as the use of rubber dam isolation, satisfactory coronal restoration, and appropriate follow-up of endodontically treated teeth.[83] Thus, through the years, certain concepts have come into practice and have bolstered the successful practice of endodontics today. These include: reduction of bacteria through effective canal debridement, sufficient seal of the root canal system using three-dimensional obturation, adequate coronal restoration to minimize coronal leakage, and appropriate long term maintenance.

MECHANICAL INSTRUMENTATION

The first phase of endodontic therapy is instrumentation and this entails enlarging the root canal space adequately for proper delivery of irrigation and disinfection.[81,84, 85] It is imperative to clean and shape while maintaining the original shape of the canal to prevent impairment and weakening of the tooth structure and damage to the surrounding periodontium as well.[81, 86] While it has been shown that physical debridement of the canal can decrease bacterial loads dramatically, 35 percent to 53 percent of canal surfaces remain un-instrumented. [87-89] In addition, studies have shown that bacteria can infiltrate accessory and lateral canals as well as penetrate into dentinal tubules as much as 300 μm. [90, 91] Taking this into account, additional disinfection is required, which is achieved with chemical irrigation.

CHEMICAL IRRIGATION

In conjunction to mechanical debridement, chemical irrigation is paramount to the proper disinfection of root canals. The gold standard irrigation solution in endodontics is sodium hypochlorite (NaOCl), which is advocated for its ability to dissolve organic tissues, disinfect the canal space, and aid in lubrication of the canal during instrumentation [23]. At a pH of 11, NaOCl—primarily existing as hypochlorous acid—can disrupt many cellular functions such as oxidative phosphorylation and DNA synthesis as well as disturb cell membranes.[92-94]

Despite its many benefits sodium hypochlorite does not exist without shortcomings; its inability to dissolve inorganic smear layer formed during mechanical instrumentation supports the use of additional chelating agents such as EDTA.[23] Irrigation of a canal for 1 min with 17-percent EDTA has been shown to be adequate for

removal of the smear layer[95] and as a result, when combined with NaOCl the two can remove canal debris more effectively.[96] In addition to improved debridement, removal of the smear layer has been shown to enhance the sealing abilities of obturation materials.[97]

Other limitations of NaOCl are its inability to inactivate endotoxin[98] its lack of substantivity,[99, 100] and its cytotoxicity when extruded into the periradicular tissues.[23] The use of chlorhexidine gluconate (CHX) has been championed to overcome some of these shortcomings of NaOCl. Chlorhexidine has substantive effects that render it capable of reducing bacterial loads for extended periods of time through electrostatic binding to bacterial cell wall and disrupting its integrity.[101, 102] Care should be implemented when using CHX with NaOCl as the combination of the two can form a potentially harmful precipitate which has been identified as Para-chloroaniline (PCA) [103] in some studies and parachlorophenylurea (PCU) and parachlorophenylguanidyl-1,6-diguanidyl-hexane (PCGH) in others.[103, 104] Nevertheless, canals should be flushed with saline between the two irrigants to prevent the formation of this precipitate.[104]

OBTURATION

The third critical component of endodontic therapy is obturation to achieve an adequate “hermetic” seal of the canal system. Obturation materials should be biocompatible and non-irritating to the periapical tissues if extruded.[81] Studies demonstrate a higher rate of success when obturation material is dense, confined to the canal, and terminates within 1mm of the radiographic apex.[105] Finally, the use of sealers provides an appropriate seal of the canal system when obturating.[105] As seen,

endodontic therapy requires meticulous treatment and attention to detail in attaining biologic objectives which result in long-term success.

MANAGEMENT OF IMMATURE TEETH WITH NECROTIC PULP

Despite conventional root canal therapy being a fairly successful option in mature teeth with fully developed apices,[106] immature permanent teeth with necrotic pulps do not share this same success and predictability of treatment. Immature permanent teeth with pulpal necrosis generally are more difficult to treat due to specific challenges presented in the phases of disinfection and obturation of canal system. Care must be taken with open apices as irrigants can be easily extruded and filling materials overextended into the periapical tissues.[5] As a result of incomplete root formation, these teeth tend to have short roots with thin dentinal walls which increase their risk for restorative failure and root fracture.[1] Consequently, different treatment strategies have been proposed in the treatment of these complicated clinical scenarios.

APEXOGENESIS

Apexogenesis is reserved for treatment of teeth with vital pulps and open apices. Typically these teeth have a pulpitis as a result of trauma or caries. The objective in these cases is to maintain some degree of healthy, vital tissue within the canal with the hope that the root will continue maturing and develop a closed apex.[107] Clinically, shallow or a full pulpotomy is performed to remove inflamed pulp tissue. This is followed with a biocompatible dressing to cover the pulp and a final restoration. [22, 108] The size of pulpal exposure in addition to the time allowed to pass before treatment dictate the amount of tissue removal. Calcium hydroxide has been the traditional dressing used for vital pulp

therapy due to its advantages, which include rendering the tissue aseptic, biocompatibility, and stimulating new tissue formation. However, this medicament presents with disadvantages as well which include the formation of dentin that is incomplete and a basic pH ≈ 12 resulting in pulpal and tissue inflammation.

More superior pulp-capping abilities and complete dentin bridge formation without induction of inflammation have been demonstrated with the advent of MTA as a pulp-capping material.[109] This has led to superior, predictable outcomes with a greater rate of success as compared with calcium hydroxide. Despite these advantages, MTA presents with some drawbacks including an increased cost, long setting times (up to 24 hours), and discoloration of teeth.[110] Recently, other bioceramic materials such as Biodentine and other MTA derivatives have been introduced; these are all used in a similar manner for pulp capping. Similar to apexogenesis performed with calcium hydroxide, the inflamed pulp is removed partially or entirely from the pulp chamber and hemorrhage of the remaining tissues is controlled while maintaining an aseptic field. Thereafter, MTA is placed directly over the remaining vital pulp tissue, and the cavity preparation is sealed with a final restoration. Symptoms are monitored with occasional clinical and radiographic follow-ups to assess continued root development. In immature teeth with vital pulps, apexogenesis remains the preferred treatment of choice, but other alternatives exist when the pulp becomes necrotic with discontinued root development; these include apexification and regenerative endodontic procedures.

APEXIFICATION

First introduced in the 1960s, apexification attempts to create a calcified barrier across the open apex of an immature tooth with pulpal necrosis. Due to challenges faced

during obturation of a tooth with an open apex, this method was introduced to form a matrix against which obturation materials could be condensed without being expressed into the periradicular tissues. This treatment involves using long-term calcium hydroxide dressing. [8] The tooth is accessed under rubber dam isolation, working length is obtained and confirmed radiographically, and the canal is disinfected mainly via chemical irrigation. Minimal mechanical instrumentation is performed to avoid damage to the thin dentin walls. Calcium hydroxide is then placed within the canal and changed in three-month intervals until a barrier can be visualized radiographically. After the formation of an apical barrier, the canal is then obturated with MTA and/or gutta percha, and the coronal access is restored with a permanent restoration to form a final seal.[107] Treatment time with long-term calcium hydroxide can vary significantly and may span anywhere from 9 to 24 months.

Closure of the root apex using calcium hydroxide comes with a few limitations despite allowing successful obturation of the canal space. The barrier formed is typically an osteoid or cementoid material that has small remnant communication with the apical tissues with no dentin deposition to increase the root length and width.[7, 111] Patient compliance is also paramount as this type of treatment occurs over long treatment periods, and it is imperative that a disciplined follow-up regimen is followed. Finally, the use of long-term calcium hydroxide has been found to increase the risk for root fracture due to decrease in fracture resistance.[3, 112-114]

To overcome some of the shortcomings of long-term calcium hydroxide barrier technique, an artificial apical barrier can be placed apically upon which obturation material can be condensed. Disinfection of the canal space is followed with short-term

application of calcium hydroxide. Once symptoms have resolved, the canal is irrigated copiously and dried. Then, a collagen barrier is placed, and the canal is packed with a 4 to 5 mm apical MTA plug.[115] Conventional obturation with gutta percha can then be ensued without the concern for overextension into the periradicular tissues.

In omitting the use of long-term calcium hydroxide, the artificial apical barrier technique reduces treatment time and improves compliance dramatically. Thus, the tooth can be permanently restored sooner, reducing the chance for coronal leakage or worse yet, sustaining cervical fracture. Furthermore, short-term application of calcium hydroxide allows for the radicular dentin to retain more of its mechanical properties and inherent strength. MTA apical barrier apexification has been reported to have success rates ranging from 85 percent to 93.5 percent.[116, 117] However, in spite of this high success rate, an inherent weakness in this technique continues to be to its inability to increase root thickness and length through dentin deposition. The tooth remains susceptible to fracture in the future. Fortunately, with the emergence of regenerative endodontic procedures, far more promising outcomes are in sight for treatment of immature teeth with necrotic pulps.

HISTORY OF REGENERATIVE ENDODONTICS

The first concept of a regenerative endodontic procedure came in 1961 when Nygaard-Østby conducted tests whereby he elucidated tissue healing and repair in the root canal in the presence of a blood clot. In this study, seventeen teeth (from nine patients) with necrotic or vital pulps received root canal treatment whereupon the canal was reamed and the apex was enlarged; intracanal medicament was used for teeth with pulpal necrosis. Bleeding was then induced in order to partially fill the canal apically, and

the remaining canal space was obturated with kloroperka placed immediately over the blood clot. The teeth were then restored and after a follow-up period ranging from 13 days to 3.5 years, the teeth were extracted and assessed histologically. Though there were some failures likely due to leakage, most teeth exhibited resolution of inflammation in the periodontal membrane in as early as 35 days. In some cases, even radiographic evidence of apical closure was noted.[118] The apical portion of the canal was seen to contain normal vascularized fibrous connective tissue, but it lacked the structure of pulp tissue and lacked desirable cells such as odontoblasts.[21] This study was revolutionary in endodontic regeneration because it was the first to show the healing potential of a patient's own endogenous biologic tissue.

Just a few years later, in 1966, Rule and Winter published a study where they treated immature teeth and instrumented the canals short of the remaining vital tissue. They followed this with disinfection and an interappointment dressing made of a polyantibiotic mix. Their findings indicated complete resolution of symptoms of disease and the continued development of the root.[119] This was the first case report in which teeth were shown to undergo continued root development after disinfection of the root canal with polyantibiotic pastes. [21]

More recently, successful continued root development has been demonstrated in immature teeth with necrotic pulps with the use of polyantibiotic pastes as disinfectants to render the root canal aseptic. The first contemporary regenerative endodontic procedure study was published by Iwaya who used a double antibiotic paste (DAP) composed of ciprofloxacin and metronidazole in the disinfection step of treatment. [36]

In this study, Iwaya's disinfection protocol consisted of using 5% NaOCl and 3% hydrogen peroxide along with application of intracanal DAP as a medicament. After treatment spanning 6 visits, continued root development was noted, and thirty months a positive vital response was elicited.[36] Three years later, Banchs and Trope followed this study up with their own which detailed the successful treatment of and immature mandibular premolar with necrotic pulp. However, these clinicians used triple antibiotic paste (TAP) composed of ciprofloxacin, minocycline, and metronidazole.[15] The root canal in this premolar was irrigated with 5.25% NaOCl without instrumentation. TAP was then applied as an interappointment medicament for twenty eight days. After thorough removal of the TAP using saline irrigation, intracanal bleeding was induced to fill the canal with a blood clot and a restoration was placed coronal to this clot. Banchs and Trope had similar findings to those of Iwaya. They were able to achieve resolution of periapical inflammation, continued development of root length and width, and a positive vital response. The fundamental features which were found to be common amongst all of these successful early case reports were a large underdeveloped apex, young patients, minimal mechanical instrumentation, irrigation with NaOCl, antibiotic paste or calcium hydroxide interappointment medicament, and the formation of a blood clot in the empty canal space after undergoing disinfection. [120] Upon these clinical successes and trials, current regenerative endodontic methodology was founded.

Regenerative endodontic procedures aim to heal apical pathology and regenerate the pulp-dentin complex rendering the pulp vital again.[121] For an immature tooth with a necrotic pulp, this would result in restoration of pulpal function to allow for continued root development. [120] Regenerated pulp tissue should be functionally competent to form

dentin which replaces lost tooth structure. In order to do so, the regenerated pulp complex should have the following properties which would render it similar to natural pulp: vascularity and innervation, architectural make-up in terms of the existing cells and extracellular matrix, and the capacity to give rise to differentiation of odontoblasts that can deposit new dentin along the canal walls.[122] However, regeneration is not possible in the presence of microbial insult, and thus, the critical first step in pulpal regeneration is disinfection of the canal to provide an environment conducive to re-growth of tissues.[123] With achievement of a favorable environment through disinfection, three pillars of tissue-engineering must be met for endodontic regeneration – stem cells, scaffolds, and growth factors.[124, 125]

DISINFECTION

Our current understanding is that intraradicular infection is the etiology of apical periodontitis, and bacteria of both anaerobic gram-negative and gram-positive species inhabit this environment. *Fusobacterium, Dialister, Porphyromonas, Prevotella, Tannerella, Treponema, Campylobacter* and *Veillonella* are the most prevalent gram-negative bacteria present; and *Parvimonas, Fillifactor, Pseudoramibacter, Olsenella, Actinomyces, Peptostreptococcus, Streptococcus, Propionibacterium* and *Eubacterium* are the gram-positive species identified most frequently.[126] To add to this, in 2014 *Actinomyces naeslundii* was identified by Nagata et al. as the most prevalent species in primary endodontic infection of immature permanent teeth.[127]. These microbes thrive in biofilm communities along the canal walls and penetrate dentinal tubules as well.[128]

During the progression of an endodontic infection, the microbial profile changes in response to alterations in environmental conditions such as oxygen saturation and

availability of nutrients.[129] Early on, facultative bacteria dominate as the main species due to abundance of oxygen. As the disease process progresses and decreased perfusion limits the level of oxygen, an environment ideal for anaerobes is created.

The primary goal in treatment of an immature tooth with a necrotic pulp is the resolution of clinical symptoms and apical pathosis as evidenced by radiographic healing. This healing is induced by elimination of the microbial insult through disinfection, which is rudimentary to all endodontic procedures including REPs; disinfection is achieved through the use of intracanal irrigants and medicaments. The most common interappointment medicament reported in clinical cases is triple antibiotic paste – ciprofloxacin, metronidazole, and minocycline.[21] Ciprofloxacin and metronidazole prevent bacterial DNA synthesis while minocycline targets protein synthesis. *In-vitro* and *in-vivo* studies have been successful in demonstrating the effectiveness of these three combined antibiotics against bacteria from infected root canals.[33, 130]

Clinicians routinely use a pasty consistency of TAP at a concentration of 1g/mL. However, this high concentration has demonstrated damaging effects on human dental pulp stem cells[131] and human stem cells of the apical papilla.[37, 132] In an effort to overcome the negative cytotoxic effects of TAP on stem cells required for regeneration, lower concentrations, ranging from 0.1 mg/mL to 2 mg/mL, have been suggested. [37, 131, 132] *In-vitro* research has also demonstrated the detrimental effects of high concentrations of TAP on the mechanical and chemical properties of radicular dentin.[31, 112, 133]

As noted, TAP does not present without any other faults; another of its disadvantages is the severe capacity for minocycline to stain dentin.[35] This is especially crucial when treating teeth in the esthetic zone where many trauma cases are incurred.

Studies have recommended the replacement of minocycline with other antibiotics such as cefaclor, amoxicillin, or clindamycin.[21, 134] Clindamycin has demonstrated efficacy against many endodontic microbes.[135, 136] and some have advocated using modified TAP (mTAP) with this antibiotic in place of minocycline.[134] Meanwhile, to avoid tooth discoloration, others advocate eliminating minocycline all together [137] and using a double antibiotic paste (DAP) composed of ciprofloxacin and metronidazole. DAP has exhibited efficacy against endodontic pathogens.[36, 39, 138] In addition, in low concentrations, DAP has been shown to be least cytotoxic to dental pulp stem cells[40, 135] and this has sparked an interest in its use as an intracanal medicament.

Introduced in 1920, calcium hydroxide is another interappointment medicament that has shown efficacy against endodontic pathogens and their toxins; calcium hydroxide ($Ca(OH)_2$) has demonstrated the ability to inactivate lipopolysaccharide (LPS), which is critical in minimizing the inflammatory process.[29, 30, 139] Calcium hydroxide's successes are due to its inherent properties that allow it to release hydroxyl ions to create free radicals, which inhibit bacterial DNA replication and cell function. At a pH of 12.5, its alkalinity aids in denaturing structural proteins and critical cellular enzymes.[140]

$Ca(OH)_2$ has garnered much popularity in conventional endodontics due to its long, successful history; its use in regenerative endodontics is supported by the AAE as well. Aside from its antimicrobial efficacy, it has been shown to provide an environment conducive to the survival of stem cells of the apical papilla (SCAP) in a concentration of 1 mg/mL.[37, 132]

However, as reviewed earlier, using $Ca(OH)_2$ come with a few risks. It has demonstrated detrimental effects on the structure of radicular dentin by decreasing

fracture resistance and microhardness.[3, 133, 141]. Yassen et al. have also demonstrated its capacity to degrade superficial collagen in radicular dentin when applied for one to four weeks.[31] Meanwhile, other studies have addressed calcium hydroxide's inadequacy against specific endodontic pathogens such as *E. faecalis* and *P. gingivalis* biofilms.[39].

In regenerative endodontics, disinfection is achieved through the use of irrigants in addition to intracanal medicaments. Sodium hypochlorite remains the most commonly implemented endodontic irrigant due to its ability to dissolve organic tissues and eradicate microbes.[23] [142] When compared with saline alone, sodium hypochlorite has a greater effect against intracanal pathogens.[143] It has been shown that when exposed to sodium hypochlorite directly for 15 seconds, gram-negative anaerobic rods found in apical periodontitis are killed entirely.[144]. Its use in low concentrations (1.5%) has been advocated in regenerative endodontics due to its ability to dissolve necrotic tissues while minimizing vital tissue destruction.[21] However, despite its great value against microorganisms, NaOCl has shown inefficiency in the complete eradication of *E. faecalis,* a fastidious endodontic pathogen. Estrela et al. have exhibited NaOCl and chlorhexidine's inability to eradicate *E. faecalis* within the complex anatomy of the root canal system. Nevertheless, NaOCl still remains as the gold standard irrigant due to its ability to eliminate microbes and dissolve organic tissues.

Several physical properties such as concentration and temperature influence sodium hypochlorite's efficacy. Sodium hypochlorite's capacity to kill microbes and dissolve organic tissues is increased with higher concentrations and temperatures.[142, 145]. More specifically, its bactericidal quality doubles with every incremental 5 °C increase in temperature.[146] It is commonly used in concentrations of 5 percent to 6 percent in

conventional endodontics and this concentration has demonstrated efficacy and safety when used within the canal system.[147] However, other considerations must be accounted for during irrigation of a tooth with an immature apex. Irrigation of these canals must be approached with caution to avoid the extrusion of NaOCl into the periapical tissues resulting in severe tissue destruction and inflammation. In these scenarios, the use of a negative pressure irrigation system can be useful in avoiding a sodium hypochlorite accident. Furthermore, care must be taken when using NaOCl to reduce toxicity to stem cells, an essential requirement for regeneration. When considering concentration of the solution, Essner found that at an increased strength, sodium hypochlorite was more cytotoxic to stem cells when compared with lower strength solutions.[24] In addition, NaOCl reduces the differentiation potential of SCAP cells to develop into an ondontoblastic phenotype.[148] Considering these and many other published studies, the AAE has recommended using 1.5-percent NaOCl for regenerative endodontic procedures. [149, 150]

Ethylenediaminetetraacetic acid (EDTA) is another irrigant shown to have good efficacy in conventional and regenerative endodontic therapy. EDTA is a chelating agent used in routine root canal treatment to remove the inorganic component of smear layer. [151] During the instrumentation phase of treatment, smear layer, consisting of dentin debris mixed with bacteria and their toxins, occludes dentinal tubules, isthmuses, and fins. This smear layer has been found to inhibit adequate sealing of the canal with obturation materials; improved sealing of the canal can be facilitated by removal of smear layer using EDTA.[97] Adequate smear layer removal requires at least a one-minute rinse with EDTA.[107]

In regenerative endodontics, EDTA serves other functions than just smear layer removal such as its capacity to increase cell survival and allow for release of growth factors from the dentin. Trevino performed studies assessing the survival of cells after irrigation with various irrigants. Of the tested groups, EDTA alone irrigation showed the least amount of cytotoxicity compared to 6% NaOCl, and 2% CHX. The group irrigated with 6% NaOCl, 17% EDTA, and then 6% NaOCl again had a slightly increased cytotoxicity. Meanwhile, 2% CHX demonstrated the highest level of cytotoxicity, and no cells remained viable after treatment with this irrigant.[152] As a result, chlorhexidine is omitted from regenerative endodontics in order to preserve the livelihood of stem cells of the apical papilla. During irrigation with EDTA, dentinal growth factors are released from the dentin and this is believed to promote SCAP survival in REPs.[18] Yamauchi has also found that treatment of the canal with EDTA is capable of increasing attachment of newly formed mineralized tissue to the root canal walls.[153]

STEM CELLS

A critical component of regenerative endodontics is the utilization of stem cells; these types of cells have the inherent potential to differentiate into different cell lines. There are different types of stem cells and the more naïve a stem cell is the more variable its differentiation potential is. For instance, pluripotent embryonic stem cells can develop into virtually any type of human cell whereas multipotent cells can only differentiate into cells of certain lines. Stem cells are further sub-categorized according to the source they are obtained from. Allogenic stem cells are harvested from the same species. Autologous stem cells are collected from the same individual they are to be utilized in. Finally,

xenogenic stem cells are those which originate from a different species. These stem cells all have the potential for use in human pulp tissue engineering.

After disinfection, when bleeding is induced into a root canal during a regenerative procedure, stem cells are delivered along with the blood into the canal from local sites.[16] The stem cells located within the dento-alveolar complex are multipotent and can differentiate into cells and tissues found in their proximity. These may include any of the following cells: dental pulp stem cells (DPSCs), [154] periodontal ligament stem cells (PDLSCs) [155], stem cells form human exfoliated deciduous teeth (SHEDs),[156] denal follicle progenitor stem cells (DFPCs), [157] and stem cells from the apical papilla (SCAPs). [158, 159] Subsequent to exposure to growth factors, many of these cell types, such as DPSCs and SCAPs, have shown potential use in regeneration as they have demonstrated the ability to differentiate into odontoblasts, which produce dentin. [159,160] However, SCAPs have shown a greater potential for regeneration due to their superior proliferation rates compared to DPSCs. [158-160] Thus, many of the regenerative studies investigating stem cells have been focused on the cells' potential to differentiate and regenerate the dentin and pulp proper.[124].

SCAFFOLD

Scaffolds function as an extracellular matrix does whereby an environment is provided for stem cell proliferation and differentiation as well as angiogenesis.[121] In 1976, Nevins was the first to present collagen gel scaffolds that could be used in regeneration.[161] More recently, Thibodeau demonstrated the potential of a blood clot to present as a scaffold where stem cells could be mobilized to and contained.[162] This study, performed in dogs, assessed different scaffolds and their influence on

revascularization; the scaffolds studied included a blood clot, collagen scaffold, and a combination of the two in the third group. The outcome of the study indicated an increased success rate when a blood clot was used compared to a collagen scaffold alone. Thus, it was concluded that scaffolds not only create a matrix where cells can grow and differentiate within, but they contain critical components such as growth factors, which are conducive to regeneration.[162]

There are specific properties that scaffolds can possess which render them the ideal matrix. In 2001, Hutmacher identified six properties that scaffolds should have to make them inherently ideal for regenerative endodontics.[163] These are:

1. Structure which is porous for tissue and vascular integeration.
2. Biodegradable at the same rate of tissue formation
3. Allows cell attachment for differentiation and proliferation
4. Adequate mechanical properties in the implanted site
5. No adverse reaction
6. Formed into many sizes and shapes easily

Currently, the blood clot has been the type of scaffold most used in abundance during regenerative procedures while others are being tested.[121] Another similar scaffold that has demonstrated a positive impact on regeneration is platelet-rich-plasma (PRP) due, in part, to its ability to concentrate growth factors within its matrix.[152,164,165]

GROWTH FACTORS

Among having many other functions, endogenous molecules such as growth factors act as signaling molecules promoting tissue growth, maturation, healing, andrepair. Growth factors have a profound impact on the regeneration potential and

outcome of stem cells as they can direct them to differentiate into different types of tissues and cells. Many of these signaling mediators are found within the dentin matrix itself, and when exposed to the right conditions they can be released into the surrounding environment to influence the neighboring stem cells and tissues.[166] For instance, the use of EDTA in regeneration has been shown to aid in release of signaling molecules such as transforming growth factor beta (TGF-β)[25]; TGF-β promotes stem cell differentiation into odontoblasts and signals pulp tissue mineralization as well as encourages wound healing and exerts anti-inflammatory effects. Meanwhile, other growth factors such as vascular endothelial growth factor (VEGF) and bone morphogenic protein (BMP) have been identified and linked to regulatory formation of new vasculature and differentiation of odontoblasts, respectively.[166]

Regenerative endodontics is not reliant solely on endogenous molecules; exogenous compounds, which have shown potential benefit, can also be used as growth factors. One of these compounds is dexamethasone which has demonstrated an influence on human DPSCs to differentiate into cells similar to odontoblasts.[167] Similarly, simvastatin has also been shown to enhance pulpal regeneration through this same ability of DPSCs to differentiate into odontoblast-like cells.[168] Taking all of these factors into consideration, there is still much to be discovered to further understand the interaction of growth factors with surrounding tissues and their application in regeneration.

CLINICAL INDICATIONS AND DECISION-MAKING

A clinician must take several factors into consideration upon deciding the appropriate treatment of an immature tooth with pulpal necrosis and choose to treat the tooth with an apexification or a regenerative procedure; these include clinical outcomes,

patient expectations and compliance, and appropriate indications for each type of treatment. Regenerative procedures have been largely reserved for clinical treatment of teeth with pulpal necrosis, large open apices, and undeveloped roots. The outcome of REP treatment is defined by three parameters which have been set by the AAE for measurement of the success of treatment – elimination of symptoms and periradicular healing, continued root growth, and positive response to vitality testing. The positive outcome of REPs is largely based on case reports and series, which have also compared this treatment type to apexification. For instance, in one study, Jeeruphan et al. compared survival and clinical outcomes of 20 regeneration cases, 22 calcium hydroxide apexification cases, and 19 MTA apexification cases and found that REPs resulted in greater survival rates as well as better increase in root width and length.[12] Their reported survival rates for regeneration procedures, MTA apexification, and calcium hydroxide apexification were 100 percent, 95 percent, and 77.2 percent, respectively.

Despite the clinical success and sparked interest demonstrated by regenerative procedures, the quality of evidence supporting this treatment is low. Much current knowledge in regenerative endodontics comes from case reports and series. The current available evidence lacks in treatment protocol standardizations which clearly highlights the inability to analyze data from which specific, concrete deductions could be drawn.[21] Additionally, one study criticizes this available evidence due to inconsistencies in radiographic examination and angulation and discrepancies in reporting successful treatments only.[169] The modern clinician faces a dilemma in clinical decision-making when considering the unpredictable and low quality of published evidence. Nevertheless, clinicians find themselves drawn to regenerative endodontic procedures since they are an

improvement to alternative treatment protocols that leave the tooth compromised and prone to fracture.[170] The AAE has echoed this growing interest in this treatment option by outlining recommendations and guidelines for REPs using the currently available data.[150] These guidelines and growing evidence continue to provide testimony to the benefits regenerative endodontics, which emboldens clinicians to use REPs as a treatment option.

MATERIALS AND METHODS

HUMAN TEETH SELECTION

Extracted human teeth were utilized in the study and these were collected and stored in 0.1% thymol at 4°C. Certain criteria needed to be met for the teeth to be selected visually and included in the study. The inclusion criteria were: caries-free crowns and roots, complete root formation, and at least 4-mm-flat midroot diameter in the buccolingual or mesiodistal dimensions. Teeth with restorations and caries, cracks, hypocalcification, hypoplasia, and immature roots were excluded from the study.

HUMAN DENTIN SAMPLE PREPARATION

The teeth, selected according to the inclusion and exclusion criteria, were sequentially prepared into 96 dentin samples (Figure 2, Figure 3). After removal from the 0.1-percent thymol storage media, the teeth were rinsed with sterile water for 10 seconds. A water irrigated high-speed saw was used to separate the roots from the crowns, and the roots were bisected and separated further in preparation of the dentin samples (Figure 3, Figure 4). Each root was mounted onto an acrylic plate (Figure 5) and sectioned into a 4x4-mm dentin slab with a water irrigated double blade low speed saw (Figure 6). To prepare for polishing, these initial and rough dentin slabs were secured (with the cementum side facing down) onto a 38-mm cylindrical mounting block (Figure 7). A Struers Rotopol 31 polishing unit (Struers, Cleveland, OH) was used to successively polish the pulpal side (facing up) with 500-, 1200-, 2400-, and 4000-grit SiC abrasive papers (Figure 8). After polishing, the dentin samples were examined and specimens with surface defects were excluded and replaced. To remove the smear layer,

as described in the literature [171, 172], the specimens were sonicated with 1.5-percent NaOCl and 17-percent EDTA for 4 minutes. The final dimension of each dentin sample was 4x4 mm with a thickness of 1 mm to 1.5 mm. Prior to use in the study, each sample was rinsed with sterile water and wrapped separately in Whirl-pak bags (Sigma-Aldrich, St. Louis, MO, USA) with moist cotton gauze to prevent dehydration. The packaged samples were sterilized with ethylene oxide gas and stored at 4°C until further use.

ANTIBIOTIC PASTE PREPARATION

Protocols in previously published studies were used to prepare radiopaque DAP in two concentrations using minor alterations to the preparation to add a radiopacifier into the pastes.[133, 173] In summary, 1 mg/mL and 10 mg/mL concentrations of DAP were made by dissolving 25 mg and 250 mg of equal quantities of metronidazole and ciprofloxacin in 25 mL of sterile water (USP water, Corning. Manassas, VA), respectively. In order to form a 30% (w/v) of radiopaque DAP paste, 7.5 g of barium sulfate (5-µm powder, Sigma-Aldrich, St. Louis, MO) was stirred into the pastes. The final paste-like consistency of DAP was achieved by steadily incorporating 1.75 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) at room temperature. Finally, 1 mg/mL and 10 mg/mL homogenous injectable radiopaque DAP pastes free of any bubbles were obtained by centrifugation for 15 minutes at 7000 rpm.

BACTERIAL STRAINS AND MEDIA

After IRB approval (IRB# 1510640949), two clinical isolates were previously obtained as described in a study by Sassone[174] and were used in the study. These two separate clinical bacterial samples were collected from root canals of immature and

mature teeth with necrotic pulps. The first bacterial sample was collected from an immature tooth with a necrotic pulp that was being treated with a regenerative endodontic procedure. The second sample was collected from a mature tooth with a necrotic pulp that was being treated with conventional root canal therapy. The biofilm collection was conducted in the same way for both of the teeth. Rubber dam isolation was used for the teeth. After cleaning the rubber dam and tooth with 3.0-percent hydrogen peroxide and disinfecting with 6.0-percent sodium hypochlorite, sterile round burs were used to access the pulp chamber. The operatory field including the pulp chamber was then disinfected with a cotton swab soaked in 6.0-percent sodium hypochlorite. Sterile 5.0-percent sodium thiosulfate was used to inactivate the sodium hypochlorite solution. A #15 endodontic hand file with the handle removed was placed into the infected root canal to collect the samples. This file was inserted into the canal up to 1 mm from the apical foramen and for 30 seconds a filing motion was made in the canal. To wick and collect the tissue fluid in the canal, three separate sterile paper points were placed into the canal to the same working length for up to 1 minute. The file and paper points were then placed in 2 mL of brain heart infusion broth supplemented with 5 g/l yeast extract and 5% by volume vitamin K and hemin (BHI-YE) growth medium for further growth. This medium was vortexed to elute the microorganisms from the paper points and grown anaerobically for 48 hours at 37˚C and 100-percent humidity. After 48 hours of growth, these microorganisms were frozen at -80 ˚C with 10-percent sterile glycerol until their further use.

When the frozen clinical isolates of bacteria were ready for use, BHI-YE was used to grow the microorganisms in an anaerobic environment at 37°C and 100-percent

humidity. Hydrogen gas generating sachets (GasPak EZ, Becton, Dickinson and Company, Franklin Lakes, NJ) were used to remove the oxygen from the container to produce anaerobic growing conditions for the bacteria.

EXPERIMENTAL GROUPS

A total of 96 dentin specimens were utilized. Six experimental groups were formulated with 16 dentin samples in each group. In each experimental group 8 of the dentin samples were infected with the clinical isolate from an immature tooth with necrotic pulp. The second set of 8 samples was infected using the clinical isolate from a mature tooth with necrotic pulp.

The groups to be tested were as follows:

Group 1 – 1 mg/mL radiopaque DAP

Group 2 – 10 mg/mL radiopaque DAP

Group 3 – Commercial calcium hydroxide (UltraCal XS, Ultradent, South Jordan, UT, USA)

Group 4 – Placebo (methylcellulose with barium sulfate paste)

Group 5 – Bacteria in growth medium, no antimicrobial treatment

Group 6 – Growth medium without bacteria or antimicrobial treatment

Group 1 through Group 5 was inoculated with bacteria. Group 5 and Group 6 served as the control groups. Group 5 was inoculated with bacteria but did not receive any treatment. Group 6 was used as a sterile control and dentin samples were placed in sterile BHI-YE medium supplemented with vitamin K and hemin without any bacterial culture. In group 1, 1 mg/mL was used since it fell within the range of recommended concentrations for regenerative procedures according to the current AAE guidelines.

Group 2 was treated with 10 mg/mL radiopaque DAP and this was chosen as a positive control because a recent pilot study conducted at IUSD demonstrated its effectiveness in eradicating 3-week old biofilms.

BACTERIAL GROWTH ON ROOT SPECIMENS

Ethylene oxide was used to sterilize all the dentin specimens prior to use. When ready to use, the dentin specimens were placed individually with the polished pulp side facing upward into sterile 96 well plates (Figure 9). For each group of 16 wells 190 µl of sterilized BHI-YE growth media supplemented with vitamin K and hemin was placed into them (Figure 10). In groups 1 through 5, eight of the wells had 10 µl of the mature tooth clinically isolated biofilm added and the other eight wells had 10 µl of the immature tooth clinically isolated biofilm added. Group 6 received only BHI-YE growth medium with vitamin K and hemin without bacteria (n = 16). The total volume in each well was 200 µl and these dentin specimens were incubated anaerobically at 37°C and 100-percent humidity for three weeks. During the incubation period, the growth media was replaced once every week (Figure 11). After the establishment of biofilm on the dentin specimens, the experimental treatments were applied to the dentin samples in Group 1 through Group 4. The presence of established biofilms was confirmed using SEM.

HUMAN DENTIN SPECIMEN TREATMENT

After the establishment of biofilm, the dentin samples were ready to be treated as indicated by the experimental groups (Figure 12). The dentin specimens were transferred to new wells in sterile 96 well plates and supplemented with 50 µl of growth medium. The specimens in groups 1 and 2 were treated with 100 µl of 1 mg/mL radiopaque DAP

and 100 µl of 10 mg/mL radiopaque DAP, respectively. Group 3 was treated with 100 µl of $Ca(OH)_2$ intracanal medicament. Group 4 was treated with 100 µl of a radiopaque placebo paste. Groups 5 had biofilm grown on the dentin specimens but did not receive treatment. Group 6 received no bacteria or treatment. In order to maintain 100-percent humidity and prevent drying of the treatment pastes, the remaining empty wells were filled with sterile water. These 96-well plates were then be incubated anaerobically at 37°C and 100-percent humidity for a total treatment time of one week.

BIOFILM DISRUPTION ASSAYS

After a 1-week treatment of the dentin samples, sterile water was used to gently wash each dentin specimen twice to remove any non-adherent bacteria before it was transferred to a sterile plastic test tube with 200 µl of sterile water (Figure 13, Figure 14). In order to detach the microorganisms from the biofilm, each tube was sonicated for 20 seconds followed by 30 seconds of vortexing (Figure 15). 1:10 and 1:100 dilutions were made of this biofilm (Figure 16). Spiral plating of these bacterial dilutions was made on BHI-YE agar plates (CDC, BioMerieux) (Figure 17, Figure 18). These plates were incubated anaerobically for 24 hours at 37°C and 100-percent humidity (Figure 19). An automated colony counter (Synbiosis, Inc., Frederick, MD) was used to determine the number of CFUs/mL in each dilution (Figure 20, Figure 21).

STATISTICAL ANALYSIS

The effects of treatment and tooth type (mature or immature) on CFUs were evaluated using Wilcoxon Rank Sum tests. Pair-wise comparisons were made using Fisher's Protected Least Significant Differences. A 5-percent significance level was used

for all tests. The CFU data were expected to be log-normal, so a natural log transformation of the data was used in the analyses.

SAMPLE SIZE

Based on previous data, the coefficient of variation was estimated to be 0.6. With a sample size of 8 per treatment from each tooth type, the study was estimated to have 80-percent power to detect a 2.5x difference in means between any two groups, assuming two-sided tests each conducted at a 5-percent significance level.

RESULTS

BIOFILM VALIDATION

The polymicrobial nature of the mature and immature biofilms used in this study were confirmed using scanning electron microscopic images (Figure 22, Figure 23). The microorganisms grown on the dentin specimens for three weeks exhibited a polymicrobiral and heterogenous biofilm attached to the dentinal surface. The thick and varied structure of the biofilm exhibited numerous cocci and rod-shaped microorganisms that were evident in both the immature and mature samples.

DIRECT ANTIBACTERIAL EFFECTS OF TREATMENTS

The mean of the $\log_{10}$ CFU/mL values of the tested groups for the immature biofilm were as follows: 0.00 for the sterile group, 6.82 for the untreated group, 6.74 for the placebo group, 0.93 for the calcium hydroxide group, 0.00 for the 1 mg/mL DAP, and 0.00 for the 10 mg/mL DAP. The mean of the $\log_{10}$ CFU/mL values for the mature biofilm were as follows: 0.00 for the sterile group, 6.53 for the untreated group, 6.54 for the placebo group, 0.29 for the calcium hydroxide group, 0.00 for the 1 mg/mL DAP, and 0.00 for the 10 mg/mL DAP. The sterile group exhibited no bacterial growth in both the immature and mature groups. These data are summarized in Table II.

MATURE AND IMMATURE BACTERIAL COUNTS

When comparing the immature and mature species, no significant difference was found between the biofilms when they were treated with 1 mg/mL DAP, 10 mg/mL DAP, calcium hydroxide, or placebo paste. A significant difference was noted between the

biofilms in the no treatment group ($p = 0.0136$) demonstrating that in the untreated groups, the immature groups demonstrated significantly increased biofilm growth compared to mature groups. These findings are summarized in Table III.

COMPARING THE EFFECT OF TREATMENTS

When evaluating the direct antimicrobial effect of 1 mg/mLand 10 mg/mL DAP and calcium hydroxide against both types of bacterial biofilms, a significant antibacterial effect was noted compared to the control groups and the placebo paste groups ($p < 0.05$). There was no significant difference in treatment of the biofilms with 1 mg/mL or 10 mg/mL DAP, or calcium hydroxide. Both the 1 mg/ML and 10 mg/mL DAP resulted in complete eradication of the immature and mature biofilms. However, there was a trend of more growth in the calcium hydroxide groups compared to the 1 mg/mL and 10 mg/mL groups despite a lack of statistical difference. Table IV and Figure 25 summarize these findings.

COMPARING THE EFFECT OF PLACEBO PASTE

There was no significant difference in bacterial growth between the placebo paste and the untreated groups against both types of biofilms ($p > 0.05$). Bacterial biofilms obtained from the immature tooth and mature tooth exhibited similar growth when treated with placebo paste and in the untreated groups. The placebo paste did not demonstrate any antibacterial effects. Table IV summarizes this finding.

FIGURES AND TABLES

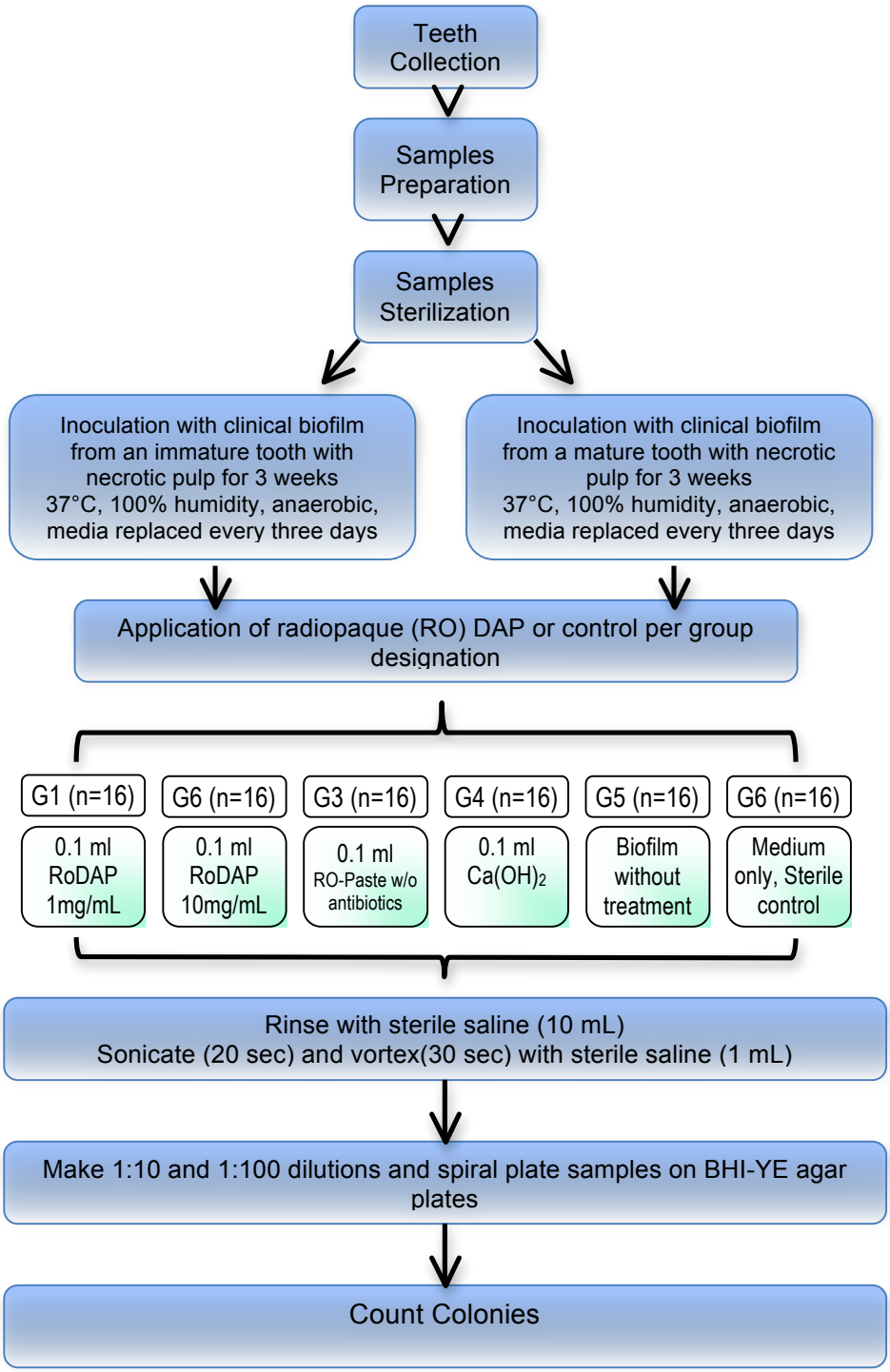

FIGURE 1. Flowchart of experimental methodology.

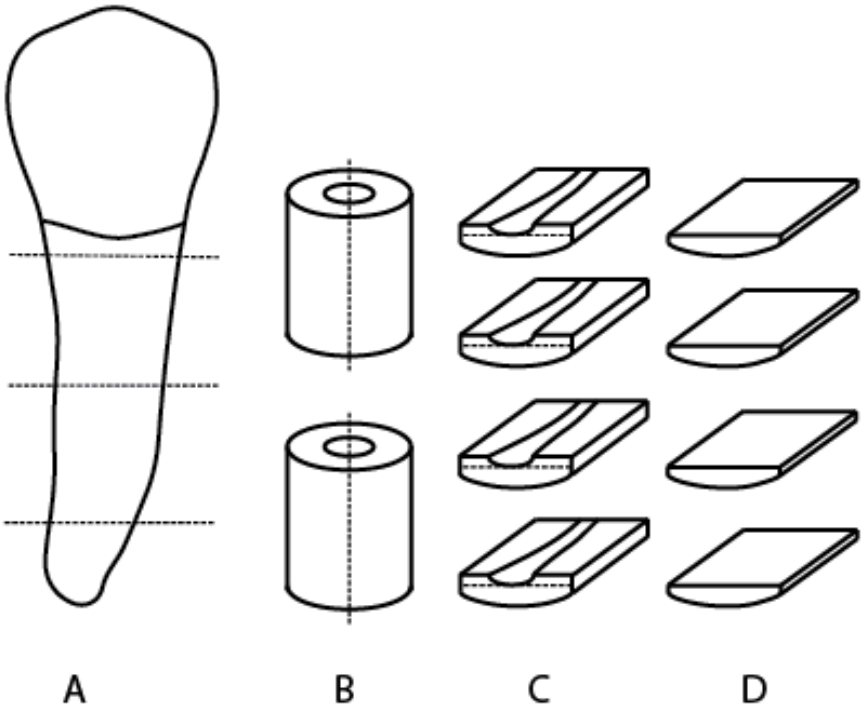

FIGURE 2. Roots (A) were sectioned, cut longitudinally (B), and polished flat on the pulpal side (C, D).

FIGURE 3. Example of sequential sectioning and final dentin sample.

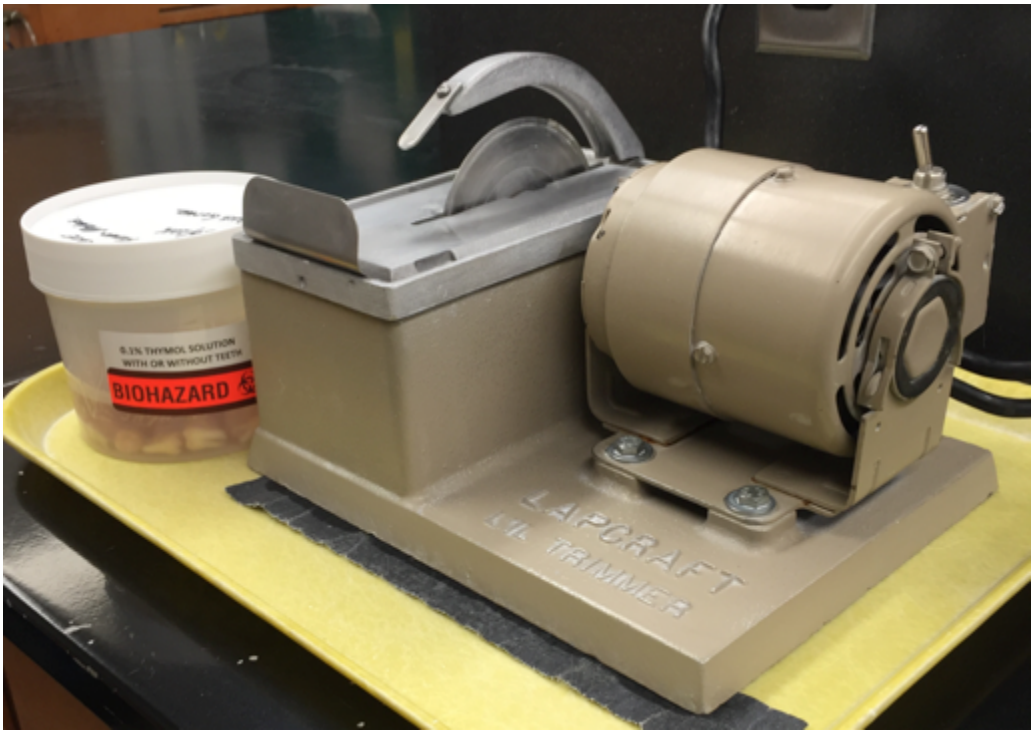

FIGURE 4. High-speed saw used to initially section whole teeth.

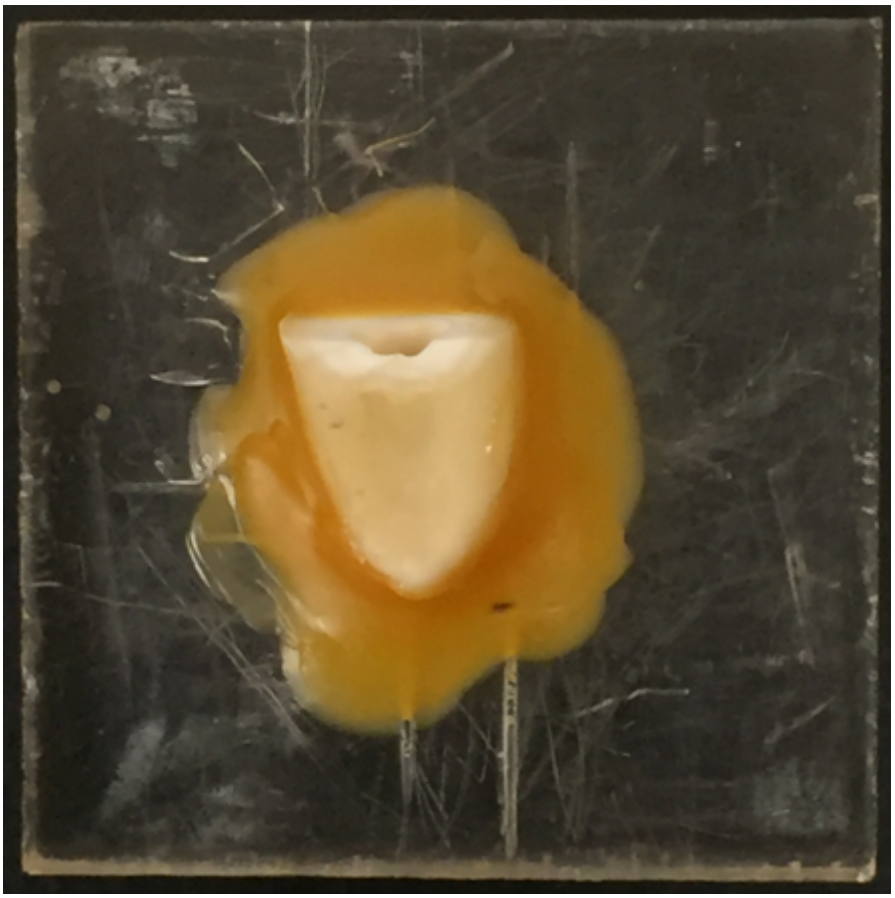

FIGURE 5. Sectioned root mounted onto acrylic plates using sticky wax.

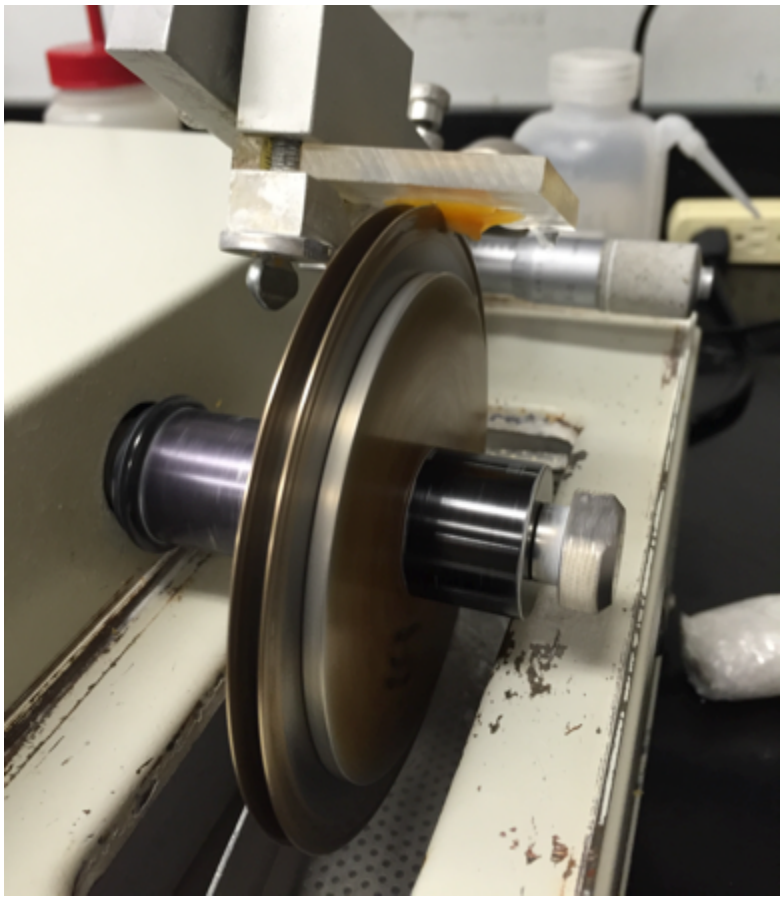

FIGURE 6. Low-speed saw used to section teeth into 4x4-mm samples.

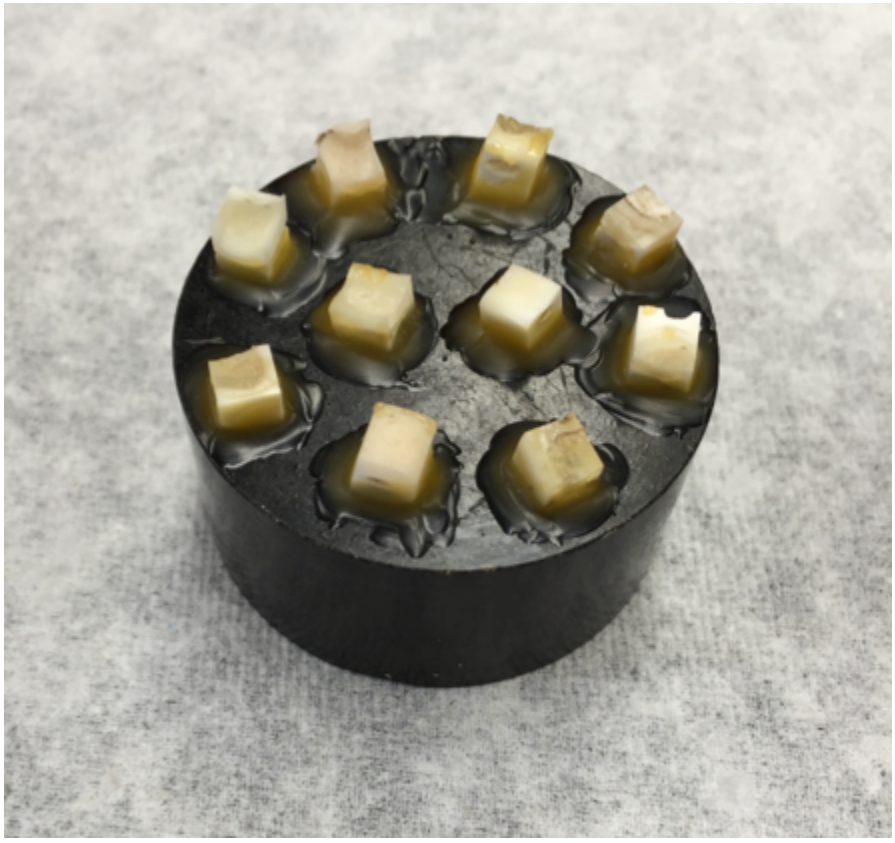

FIGURE 7. 4x4-mm dentin samples mounted on polishing jig.

FIGURE 8. Dentin polishing unit.

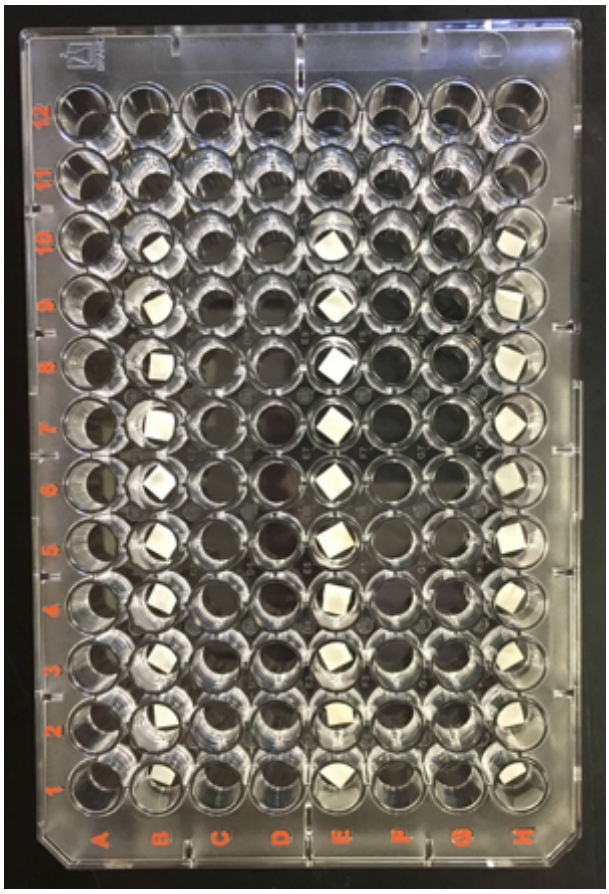

FIGURE 9. Dentin specimens placed into sterile 96-well plate.

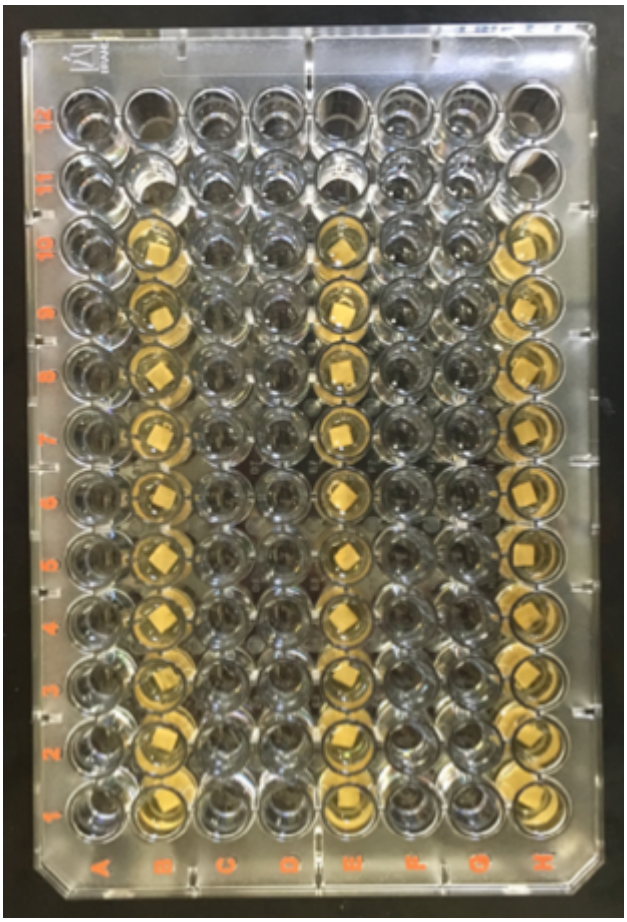

FIGURE 10. Uninoculated dentin specimens with BHI-YE broth.

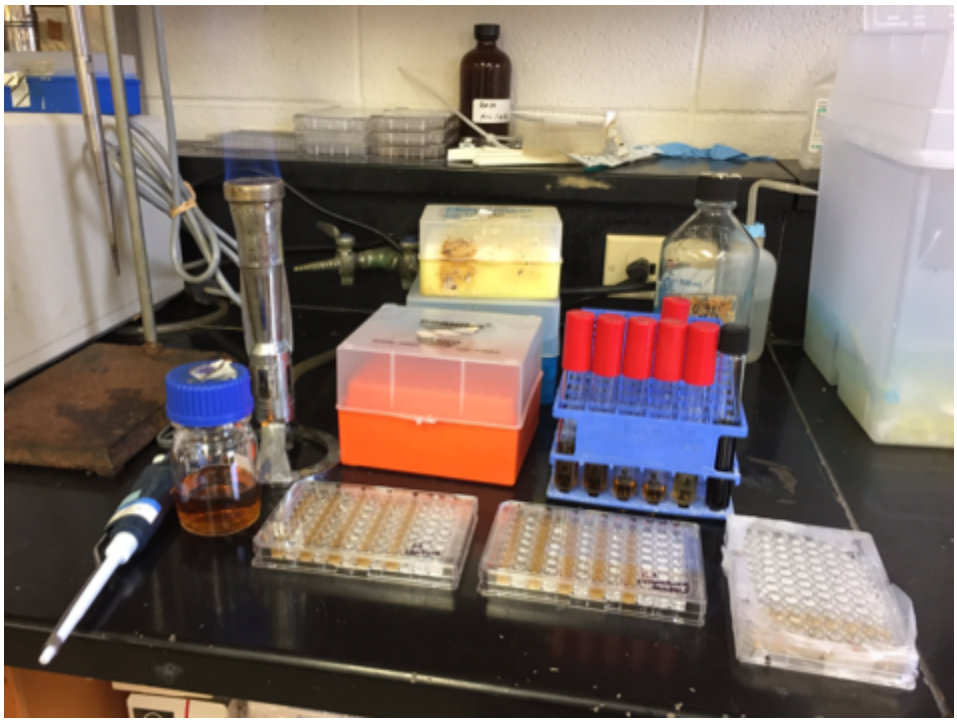

FIGURE 11. Armamentarium needed for weekly change of growth media.

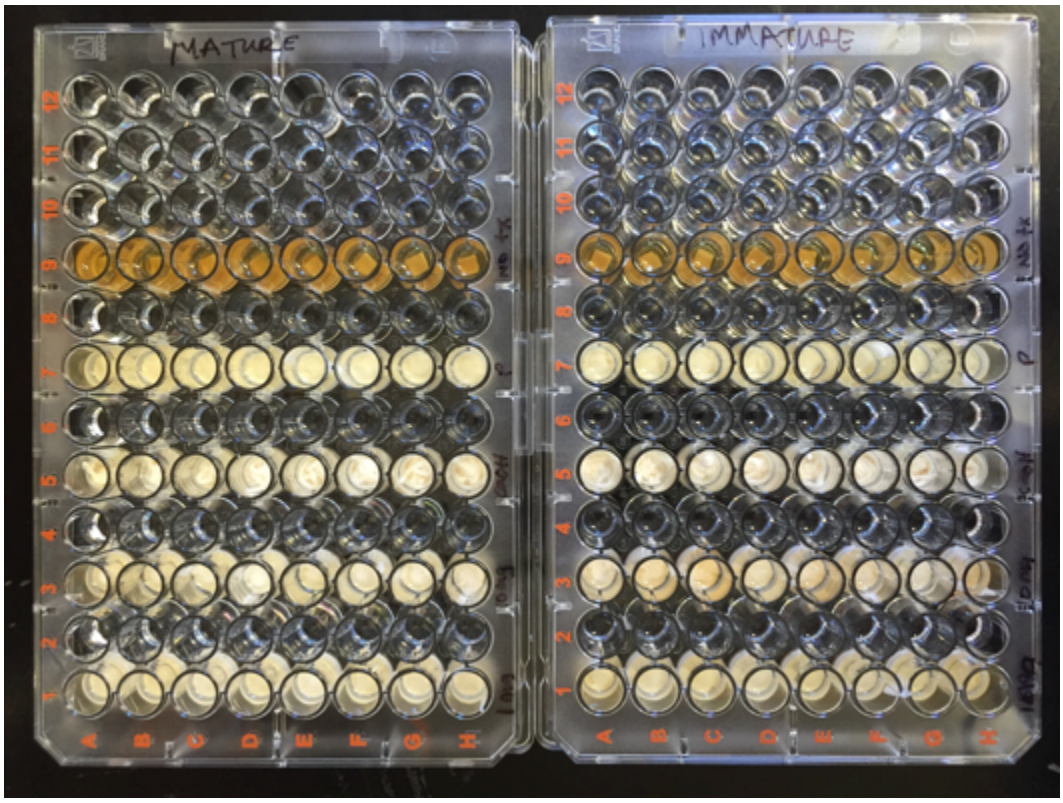

FIGURE 12. Dentin specimens containing three-week-old biofilm transferred to new 96-well plates and treated with experimental pastes.

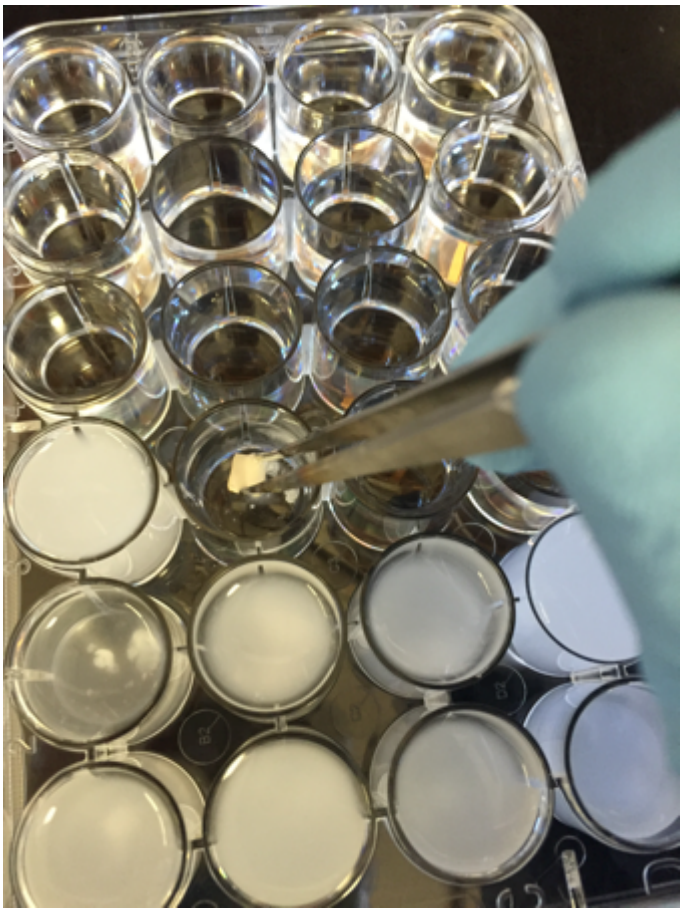

FIGURE 13. Gentle washing of dentin specimens to remove non-adherent bacteria.

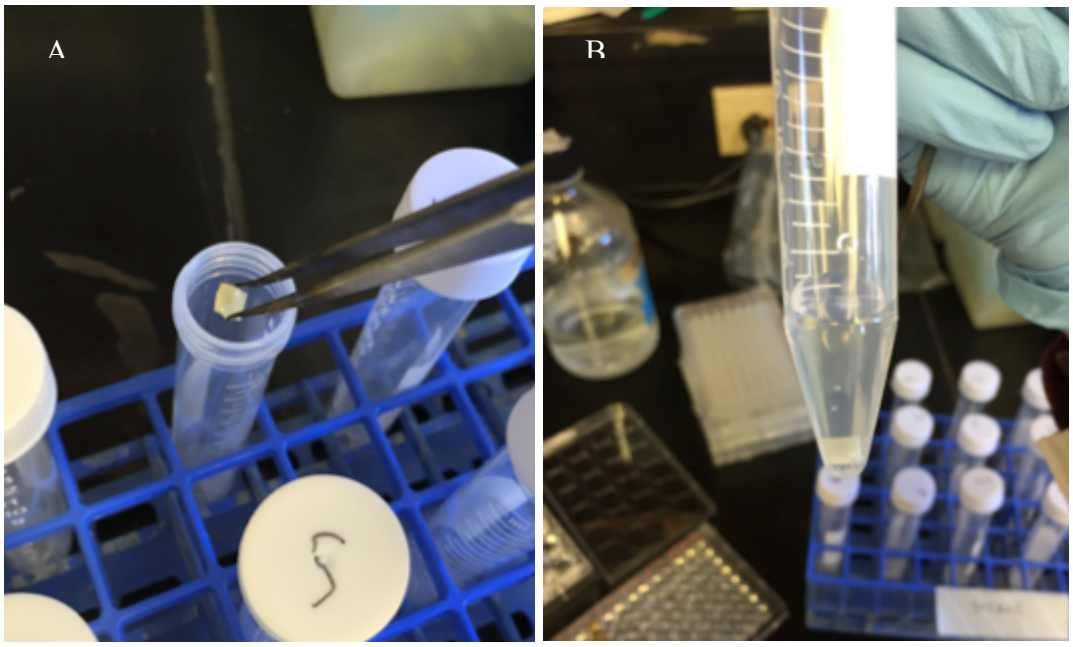

FIGURE 14. Transfer (A) of washed dentin specimens containing 3-week old biofilm to new sterile plastic tubes (B).

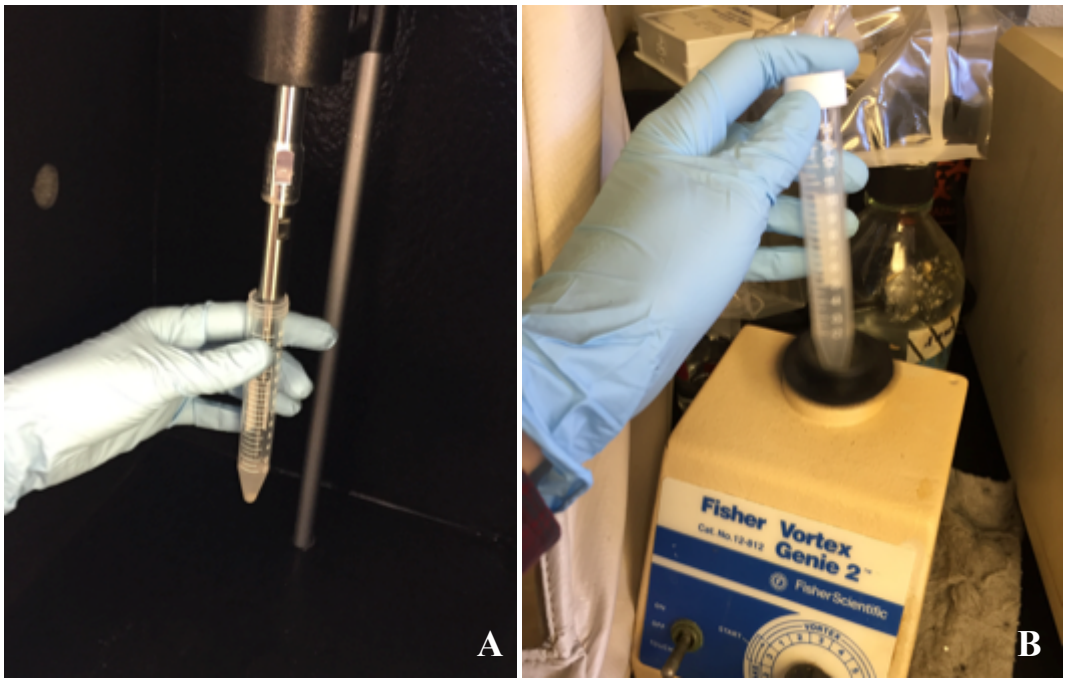

FIGURE 15, Sonication (A) and vortexing (B) for elution of biofilm microorganisms from dentin specimens.

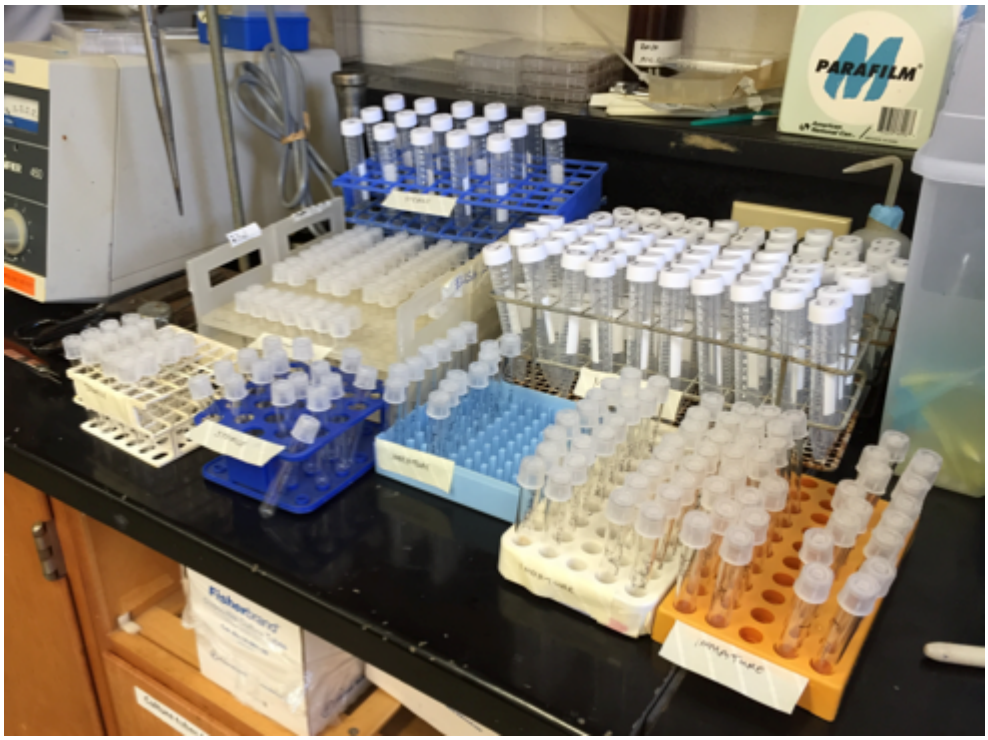

FIGURE 16. Dilution tubes for enumeration of biofilm bacteria.

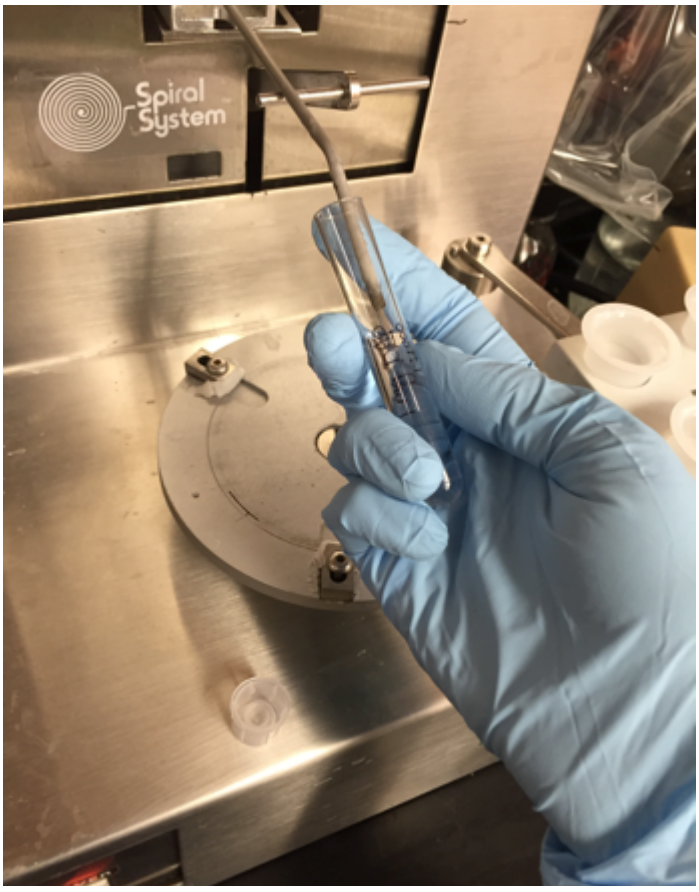

FIGURE 17. Aspiration of bacterial sample into spiral plater.

FIGURE 18. Spiral plating of biofilm bacteria onto blood agar plates.

FIGURE 19. Blood agar plates incubated in anaerobic chambers with gas paks.

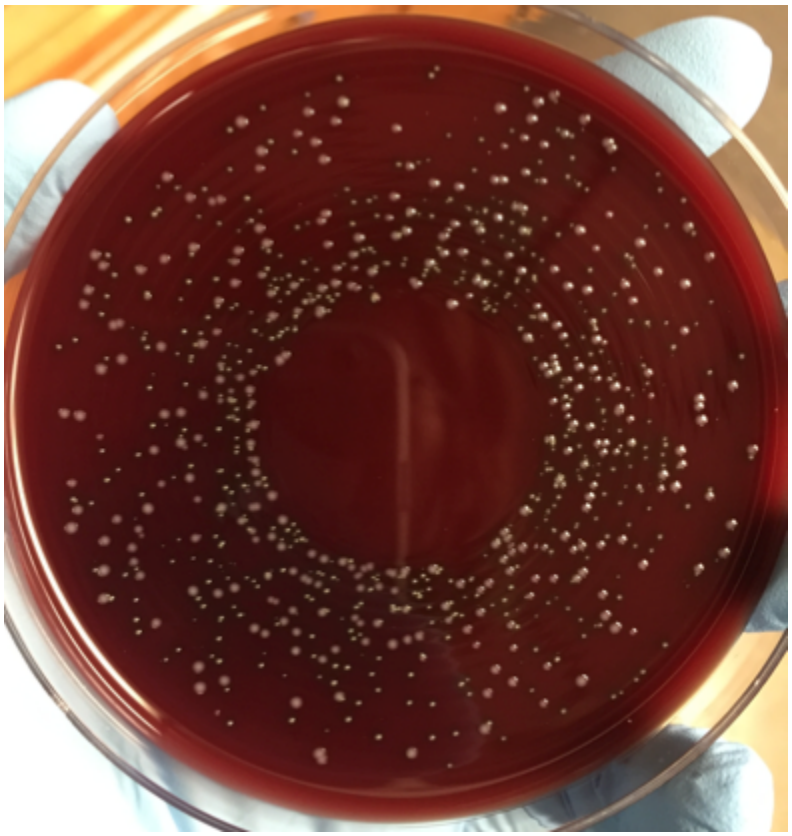

FIGURE 20. Example of bacterial growth from immature biofilm without treatment, 1:1000 dilution.

FIGURE 21. Digital colony counter.

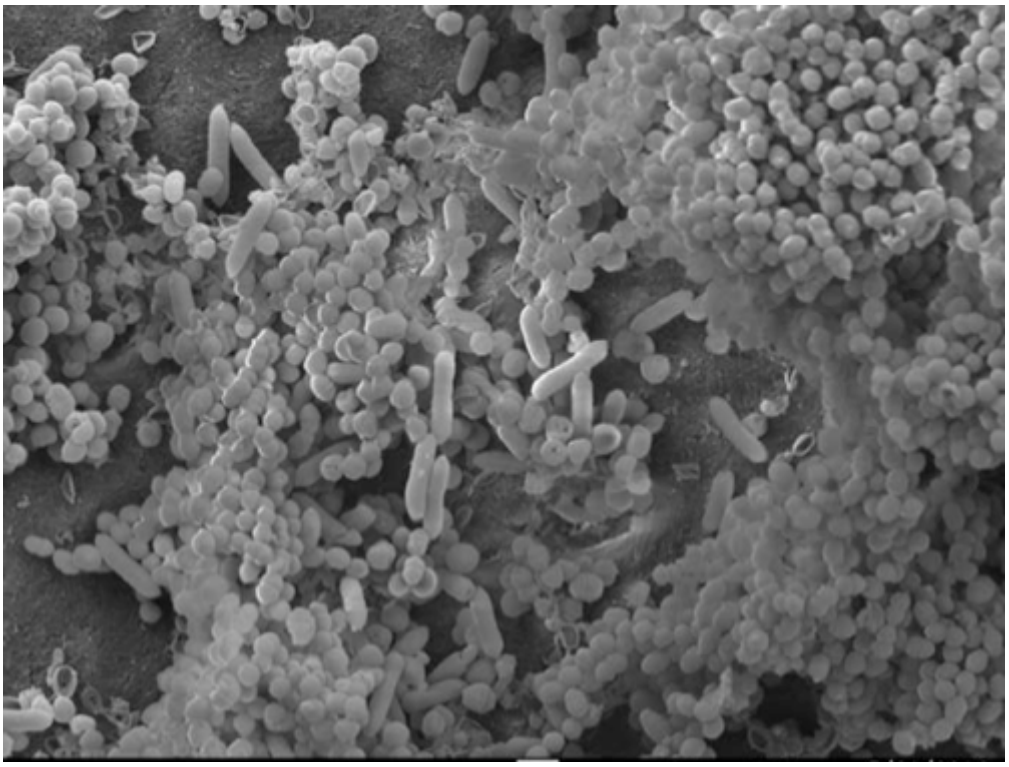

FIGURE 22. Scanning electron microscopic image of 3-week old bacterial biofilm formed by bacteria obtained from an infected root canal of a mature tooth with necrotic pulp.

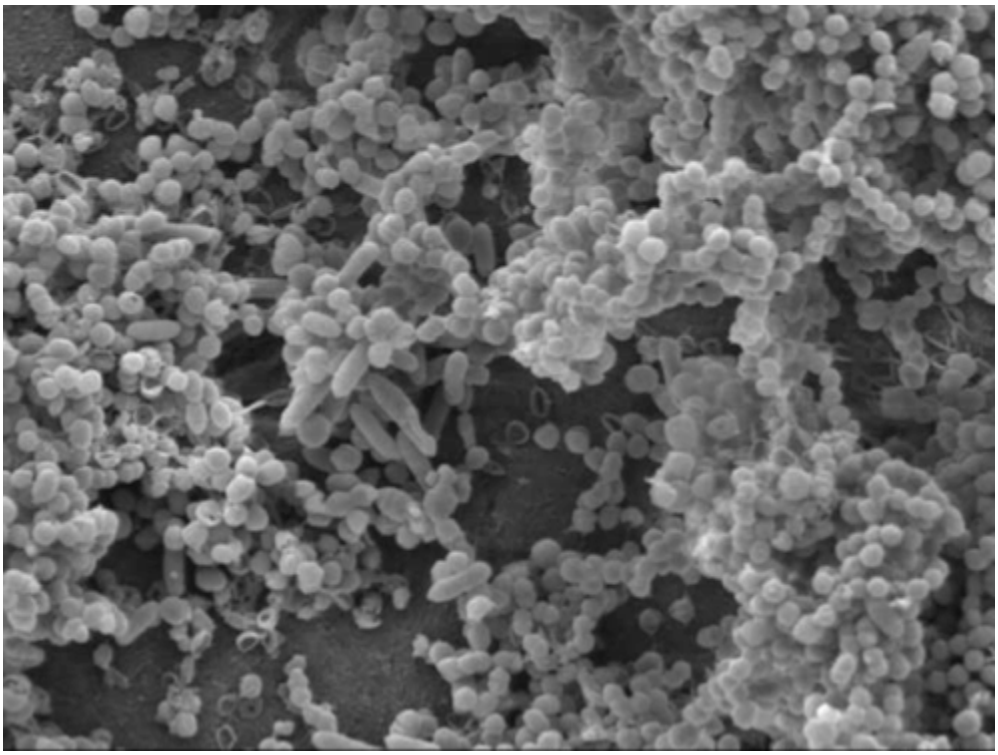

FIGURE 23. Scanning electron microscopic image of 3-week old bacterial biofilm formed by bacteria obtained from an infected root canal of an immature tooth with necrotic pulp.

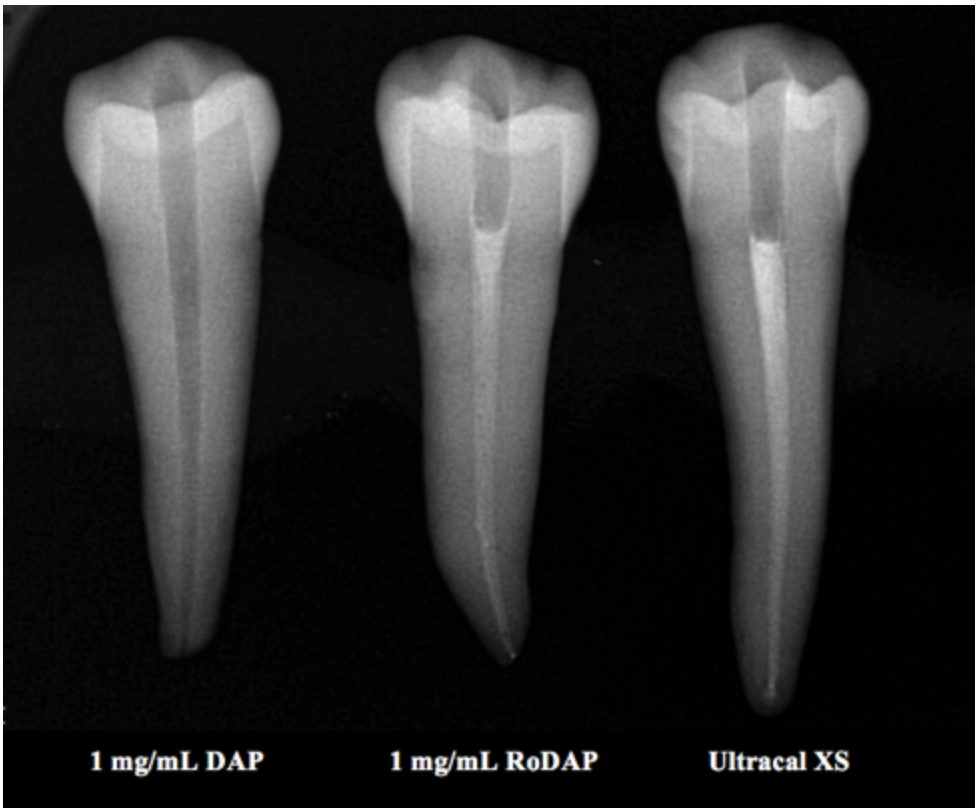

FIGURE 24. Radiograph demonstrating similar radiopacity of 1 mg/mL RoDAP compared with Ultracal XS when 30% w/v barium sulfate was added to DAP as a radiopacifying agent.

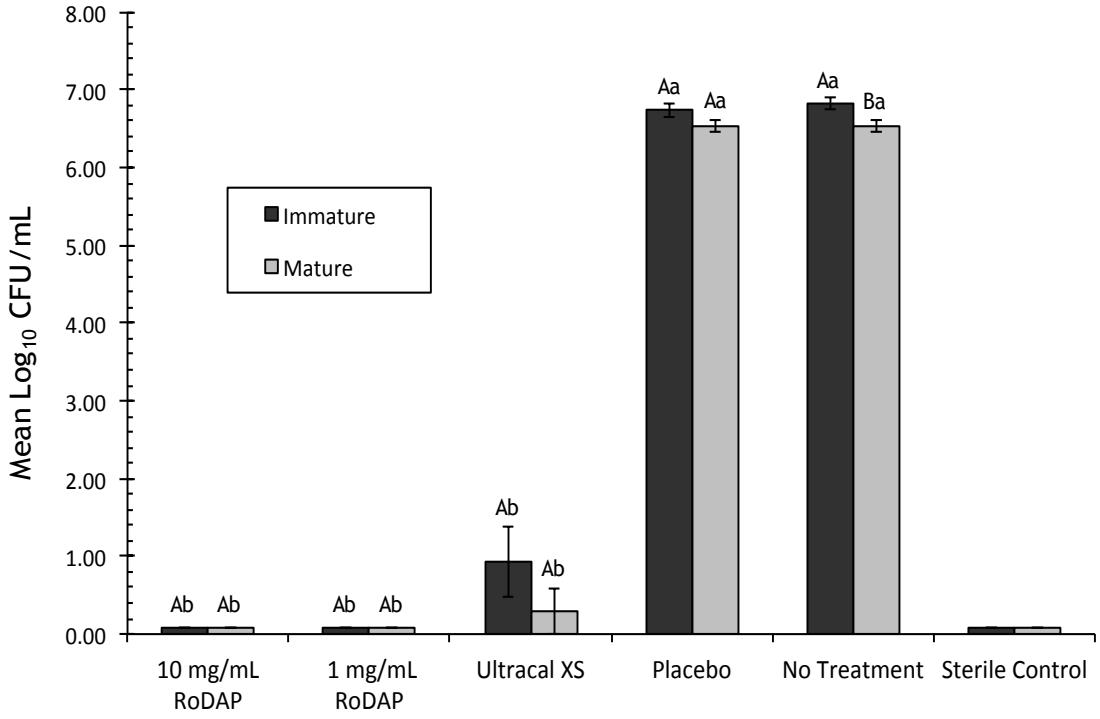

FIGURE 25. Graphical representation of data demonstrating the direct antibacterial effects of the tested radiopaque medicaments against bacterial biofilms from mature or immature teeth with pulpal necrosis represented as the mean (+SE) of the $\log_{10}$ CFU/mL.

*Different upper case letters indicate significant differences between biofilms from mature and immature teeth within the same type of treatment. Different lower case letters indicate significant differences between the different types of treatment within each type of biofilm.

TABLE I

The antibacterial effects of radiopaque and non-radiopaque 1 mg/mL DAP represented as the mean (standard deviation) of the log_{10} colony-forming unit/mL, [n= 3 per group]

Type of treatment	**Mean log_{10} (SD)**
1 mg/mL radiopaque DAP	0
1 mg/mL non-radiopaque DAP	0
No treatment	5.7 (0.03)

TABLE II

The direct antibacterial effects of the different radiopaque medicaments against bacterial biofilms from mature or immature teeth with pulpal necrosis represented as the mean (SE) of the log_{10} CFU/mL

Type of treatment	**Biofilms from immature tooth Mean log_{10} (SE)**	**Number of positive samples**	**Biofilms from mature tooth Mean log_{10} (SE)**	**Number of positive samples**
10 mg/mL RoDAP	0.00 (0)Ab	0/8	0.00 (0)Ab	0/8
1 mg/mL RoDAP	0.00 (0)Ab	0/8	0.00 (0)Ab	0/8
Ultracal XS	0.93 (0.45)Ab	2/8	0.29 (0.29)Ab	1/8
Placebo RO Paste	6.74 (0.09)Aa	8/8	6.54 (0.08)Aa	8/8
No treatment	6.82 (0.07)Aa	8/8	6.53 (0.07)Aa	8/8
Sterile control	0.00 (0)	0/8	0.00 (0)	0/8

*Different upper case letters indicate significant differences between biofilms from mature and immature teeth within the same type of treatment. Different lower case letters indicate significant differences between the different types of treatment within each type of biofilm.

TABLE III

Comparing the mature vs. immature biofilm effect for each treatment

Type of treatment	**p-value**
10 mg/mL RoDAP	1.0
1 mg/mL RoDAP	1.0
Ultracal XS	0.0831
Ro Placebo paste	0.2673
No treatment	0.0136*
Sterile control	1.0

*Significant difference between the biofilms isolated from mature and immature teeth within that experimental group, ($p < 0.05$).

TABLE IV

Comparing the effect between treatment types for immature and mature biofilm

Treatment 1	Vs.	Treatment 2	Immature Biofilm p-value	Mature Biofilm p-value
1 mg/mL		10 mg/mL	1.0	1.0
1 mg/mL		Placebo	0.0004*	0.0004*
1 mg/mL		Ultracal XS	0.0760	0.3816
1 mg/mL		No Treatment	0.0004*	0.0004*
1 mg/mL		Sterile	1.0	1.0
10 mg/mL		Placebo	0.0004*	0.0004*
10 mg/mL		Ultracal XS	0.0760	0.3816
10 mg/mL		No Treatment	0.0004*	0.0004*
10 mg/mL		Sterile	1.0	1.0
Placebo		Ultracal XS	0.0008*	0.0006*
Placebo		No Treatment	0.5635	1.0
Placebo		Sterile	0.0004*	0.0004*
Ultracal XS		No Treatment	0.0008*	0.0006*
Ultracal XS		Sterile	0.0760	0.3816
No Treatment		Sterile	0.0004*	0.0004*

*Significant difference between the two treatment types within that biofilm group, ($p < 0.05$).

DISCUSSION

In recent decades, regenerative endodontics has revolutionized the way in which immature teeth with pulpal necrosis are managed. Disinfection of the root canal space through mechanical and chemical means is imperative in producing an environment that is conducive to regeneration of host tissues within the tooth. The large canals, thin dentinal walls, and blunderbuss shape of the apical foramen in these teeth render mechanical disinfection near impossible. Thus, chemical disinfection is essential and remains the primary mode by which the microbial load is reduced to allow for regeneration to take place.

The current guidelines recommended by the American Association of Endodontists advocate the use of antibiotic pastes or calcium hydroxide for chemical disinfection. Traditionally, triple antibiotic paste has been used an intracanal medicament in regenerative endoodntics.[32, 33, 175] More recently, however, double antibiotic paste has gained much popularity over TAP due to its minimal tooth discoloration through the elimination of minocycline[137]while maintaining an antibacterial effect against endodontic pathogens. [39, 40, 176] Thus, low concentrations of RoDAP were chosen as experimental groups in this study, and their effect on multi-species biofilms was assessed as compared with previous studies, which have focused on higher concentrations.

One major limitation in using DAP is its lack of radiopacity and thus the inability to radiographically confirm its adequate placement within the entirety of the canal. In addition, during a regenerative procedure in a tooth with an open apex, over or under application of non-radipaque DAP can easily go unnoticed leading to potential

deleterious effects. To overcome this drawback, our study aimed to make DAP radiopaque using a radiopacifying compound and to test its effectiveness against bacterial biofilms from mature and immature teeth with necrotic pulps. Many different radiopacifying agents are commonly used in dental materials, and these are typically insoluble salts of heavy metals such as barium, zirconium, bismuth, and zinc among many others. Barium sulfate ($BaSO_4$) was chosen in our study due to its common application in various endodontic materials including medicaments, root canal sealers, and gutta percha filling. Namely, this radiopacifying agent was also used to compare our RoDAP groups specifically to commercially available calcium hydroxide, which also contains $BaSO_4$. When added to Portland cement, $BaSO_4$ has demonstrated that it is adequately opaque allowing its distinction from dentin and any adjacent anatomical structures.[177-179] Insoluble $BaSO_4$ is used in modern medicine due to its inexpensive and nontoxic qualities.[46] Studies have shown that $BaSO_4$ is not cytotoxic to mouse fibroblasts[180] and does not induce DNA damage to murine embryonic fibroblasts when these cell lines are exposed to it.[181]. One study even demonstrated a slight antimicrobial activity of barium sulfate when it is added to calcium hydroxide.[177] In contrast, however, our study demonstrated no antimicrobial effect when barium sulfate in a methylcellulose vehicle was tested against biofilms from mature and immature teeth with necrotic pulps. Thus, barium sulfate was added to DAP and this radiopaque antibiotic paste was tested to determine whether its antimicrobial effect would be altered compared to previous published studies.

One important aspect to note in this study is the use of methylcellulose as a delivery vehicle for RoDAP. Previously, antibiotic pastes were hand mixed with sterile

until the desired consistency was achieved. This resulted in higher concentration pastes, which as mentioned previously, can be detrimental to stem cell survival. The poor handling characteristics of low-concentration antibiotic medicaments are a deterrent to clinicians. To overcome this challenge, we used methylcellulose hydrogel to increase the viscosity of the paste and provide a stable suspension, which could deliver low concentration antibiotics within the canal more predictably. Methylcellulose is currently used with calcium hydroxide and its use has been recommended to create a paste that can diffuse more readily with slower ionic dissolution, more durability, and increased longevity of the alkaline nature of this medicament.[182, 183] [184] In addition, previous studies have demonstrated that low concentration antibiotics loaded into a methylcellulose hydrogel did not have an altered antimicrobial effect and did not cause detrimental effects on proliferation and attachment of dental pulp stem cells.[176, 185] Given these promising findings, we implemented methylcellulose hydrogel with low concentration radiopaque DAP and it demonstrated ease of delivery and predictability of placement.

The challenge with using intracanal medicaments for regenerative procedures is obtaining the proper balance of canal disinfection and minimal cytotoxicity to stem cells. The current study showed that both 1 mg/mL and 10 mg/mL RoDAP as well as calcium hydroxide provided a substantial direct antimicrobial effect against clinical bacterial isolates from mature and immature teeth with necrotic pulps. To date, based on the existing evidence, the American Association of Endodontists advocates the use of calcium hydroxide or TAP or DAP in concentrations ranging from 0.1 mg/mL to 1 mg/mL for the disinfection step of regenerative endodontics.[150]. Considering the

available evidence and the recommendations advocated by the AAE, 1 mg/mL radiopaque DAP was chosen as an experimental group as it has demonstrated better antimicrobial effects against established biofilm compared to the use of 0.1 mg/mL DAP [40]. This is in agreement with other studies which have demonstrated the effectiveness of 1 mg/mL DAP against mature biofilms.[138, 176] In these same studies, however, 1 mg/mL DAP was unable to completely eradicate established biofilms.[138, 176] By contrast, our study found that treatment of three-week-old biofilms with 1 mg/mL RoDAP resulted in complete elimination of microbes from a mature and immature tooth with pulpal necrosis. The effectiveness of 1 mg/mL RoDAP in this study was investigated to compare its effect in relation to the higher concentration of 10 mg/mL DAP which has been shown to be efficient in complete eradication of biofilms of known endodontic pathogens. [40] Generally, the direct antimicrobial effect of antibiotic pastes is concentration dependent. However, aside from the antimicrobial effect, we must consider another crucial aspect of regenerative endodontics when evaluating the efficacy of intracanal medicament, and their biocompatibility and effect on stem cells. Studies have shown that higher concentrations of DAP can be detrimental to the survival of stem cells of the apical papilla (SCAP). Specifically, 10 mg/mL has been shown to significantly reduce SCAP survival.[37] Contrastingly, other studies have shown that 1 mg/mL DAP did not negatively affect the proliferation of dental pulp stem cells or the survival of SCAP. [41, 132, 185] Considering these findings, 1 mg/mL RoDAP was shown to be just as effective as non-radiopaque DAP as reported in previous studies, but its effect on the survivability of stem cells must be explored in future studies.

The use of calcium hydroxide in regenerative endodontics has been favored by some. This present study demonstrated that calcium hydroxide was effective against clinical bacterial biofilms regardless of their source and this was statistically significant. However, when compared to RoDAP, calcium hydroxide tended to show a trend of some bacterial growth which is in contrast with a previous study. [176] One possible explanation for this is the potential for washout of the calcium hydroxide during transportation of the well-plates. Considering this finding, one of the limitations of using calcium hydroxide is its lack of continued residual antimicrobial effect when it is washed away which is unlike DAP as evidenced by previous studies. [176, 186] This is important to note since it may render the canal inadequately disinfected and any residual bacteria can regrow within the unfilled root canal system in a regeneration case. Compared to calcium hydroxide, RoDAP has the added benefit of having a lasting effect, which may give the regenerating tissues an opportunity to establish their own immune responses and manage any residual infection.

When comparing the immature and mature species, no significant difference was found between the biofilms when they were treated with any of the experimental groups. However, a significant difference was noted between the two types of biofilms demonstrating an increase in biofilm growth of the immature groups compared to the mature. This is in agreement with a previous study conducted by Jacobs et al., which also found that the direct and residual effect of DAP was reduced in the immature groups. [176] Our study did not find any difference between the mature and immature biofilms and the treatment they received. However, it can be suggested that a more vigorous biofilm from an immature tooth may have the potential to be more resistant to disinfection than

that from a mature tooth. This can further be explained by the difference in the bacterial profile of infected canals of mature and immature teeth. One recent study identified *A. naeslundii* and *Porphyromonas endodontalis* as the two species encountered most often in immature teeth with necrotic pulps[127], which are different from the *Fusobacterium* and *Prevotella* species commonly found in necrotic pulps of mature teeth.[187] The difference in the bacterial makeup of infected canals in mature and immature teeth attests to the importance of testing antimicrobial pastes against these diverse biofilms, and this validates the use of clinical isolates from two sources in our study.

It is worth nothing that other antibiotic agents exist as medicaments to disinfect root canals, and these have been applied in several other treatment situations such as conventional root canal therapy or in trauma cases. Antibiotic medicaments such as Ledermix (composed of triamcinolone acetonide and demeclocycline hydrochloride) have been frequently used after severe luxation injuries due to good antibacterial and antiresorptive qualities.[188] However, Ledermix has a high potential for staining teeth because it contains a tetracycline derivative.[110] Odontopaste, which contains clindamycin derivatives instead, has been introduced as a non-staining alternative to Ledermix. Currently, these medicaments have not been used in regenerative endodontic extensively. Some of their limitations include the potential for staining, unavailability in the U.S. market, and a lack of radiopacity. By contrast, DAP has a positive track record that supports its use in regenerative endodontics, and when it is made radiopaque, it has the added benefit of radiographic confirmation within the canal.

Some limitations exist in this study, and one of these is source of the clinical isolates. The bacterial samples were collected from a single patient in each bacterial

group, mature and immature. For this reason, any heterogeneity amongst the two types of biofilms could be attributed to natural differences between each clinical case without actual relation to the apical maturity of the teeth. A wide range of microbial diversity has been noted from tooth to tooth in previous studies.[189] Thus, further studies with an increase in bacterial samples need to be conducted to confirm the findings of this study. Another drawback of the study is the manner by which the bacterial samples were collected. Despite maintaining anaerobic conditions during bacterial isolation and treatment times, it is important to note the limitation of the study with respect to the composition of the clinical isolates. It is possible to speculate that the samples that were isolated from the canal space most likely eliminated obligate anaerobes as these species cannot survive when exposed to oxygen. Future studies should be aimed at maintaining an anaerobic environment throughout the entire experiment. Further studies testing more specific concentrations between 0.1 mg/mL and 1 mg/mL RoDAP and increased treatment times up to four weeks are warranted as well.

SUMMARY AND CONCLUSIONS

In summary, the purpose of this study was to investigate the antibacterial effect of 1 mg/mL and 10 mg/mL radiopaque DAP on radicular dentin infected with clinical bacterial isolates from mature and immature teeth with necrotic pulps. This goal was sought to further our understanding of radiopaque antibiotic medicament that can be implemented in regenerative endodontic procedures. The results found in this study show that 1 mg/mL DAP, 10 mg/mL DAP, and $Ca(OH)_2$ demonstrated clinically significant antimicrobial effects against the biofilms used in this study. Thus, our null hypothesis stating that 1 mg/mL and 10 mg/mL concentrations of radiopaque DAP will not have significant antibacterial effect against bacterial isolates regardless of the source was rejected. The second null hypothesis – that all tested concentrations of radiopaque DAP will demonstrate similar antibacterial effects against bacterial isolates obtained from immature and mature teeth with necrotic pulps – was not rejected. However, there was a significant difference found between the growth of the biofilms from the mature tooth compared with the immature tooth with an increase in growth seen in the biofilm originating from the immature tooth. Thus, it is plausible to speculate that biofilms in immature teeth may require an increased concentration of antibiotic medicament or increased treatment time to eradicate them.

REFERENCES

1. Hargreaves KM, A Diogenes, and FB Teixeira, Treatment options: biological basis of regenerative endodontic procedures. J Endod 2013;39:S30-S43.

2. Cvek, M., Prognosis of luxated non-vital maxillary incisors treated with calcium hydroxide and filled with gutta-percha. A retrospective clinical study. Endod Dent Traumatol 1992;8(2):45-55.

3. Andreasen, JO, B Farik, and EC Munksgaard, Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dent Traumatol 2002; 18(3):134-7.

4. Hatibović-Kofman, Š., et al. Fracture resistance and histological findings of immature teeth treated with mineral trioxide aggregate. Dent Traumatol 2008; 24(3): 272-6.

5. Trope M. Treatment of the immature tooth with a non-vital pulp and apical periodontitis. Dent Clin North Am 2010;54(2):313-24.

6. Frank A.L., Therapy for the divergent pulpless tooth by continued apical formation. J Am Dent Assoc 1966;72(1):87-93.

7. Steiner JC, PR Dow, and GM Cathey. Inducing root end closure of nonvital permanent teeth. J Dent Child 1968;35(1):47-54.

8. Steiner JC and HJ Van Hassel, Experimental root apexification in primates. Oral Surg Oral Med Oral Pathol 1971;31(3):409-15.

9. Dominguez Reyes, AL Munoz and TA Martin. Study of calcium hydroxide apexification in 26 young permanent incisors. Dent Traumatol 2005;21(3):141-5.

10. Soares J, et al. Calcium hydroxide induced apexification with apical root development: a clinical case report. Int Endod J 2008;41(8):710-9.

11. Shabahang S, et al. A comparative study of root-end induction using osteogenic protein-1, calcium hydroxide, and mineral trioxide aggregate in dogs. J Endod 1999; 25(1):1-5.

12. Jeeruphan T, et al. Mahidol study 1: comparison of radiographic and survival outcomes of immature teeth treated with either regenerative endodontic or apexification methods: a retrospective study. J Endod 2012; 38(10):1330-6.

13. Fusayama, T. and T. Maeda, Effect of pulpectomy on dentin hardness. J Dent Res 1969; 48(3):452-60.

14. Langer R. and JP Vacanti.Tissue engineering.Science 1993;260(5110):920-6.

15. Banchs, F. and M. Trope, Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod 2004;30(4):196-200.

16. Lovelace, T.W., et al., Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. J Endod 2011;37(2):133-8.

17. Nakashima, M. and A. Akamine, The application of tissue engineering to regeneration of pulp and dentin in endodontics. J Endod 2005; 31(10):711-8.

18. Galler, K.M., et al., Dentin conditioning codetermines cell fate in regenerative endodontics. J Endod 2011; 37(11):1536-41.

19. Roberts-Clark, D.J. and A.J. Smith, Angiogenic growth factors in human dentine matrix. Arch Oral Biol 2000;45(11):1013-6.

20. Nagy, M.M., et al., Regenerative potential of immature permanent teeth with necrotic pulps after different regenerative protocols. J Endod 2014; 40(2):192-8.

21. Diogenes, A., et al., An update on clinical regenerative endodontics. Endodontic Topics 2013;28(1): 2-23.

22. Diogenes, A.R., et al., Translational science in disinfection for regenerative endodontics. J Endod 2014;40(4, Supplement):S52-S57.

23. Zehnder, M., Root canal irrigants. J Endod 2006;32(5):389-98.

24. Essner, M.D., A. Javed, and P.D. Eleazer, Effect of sodium hypochlorite on human pulp cells: an in vitro study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112(5): 662-6.

25. Zhao, S., et al., Ultrastructural localisation of TGF-beta exposure in dentine by chemical treatment. Histochem J 2000;32(8):489-94.

26. Begue-Kirn, C., et al., Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast in vitro. Int J Dev Biol 1992;36(4):491-503.

27. Bystrom, A., R. Claesson, and G. Sundqvist, The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. Endod Dent Traumatol 1985;1(5):170-5.

28. Sjogren, U., et al., The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991;24(3):119-25.

29. Safavi, K.E. and F.C. Nichols, Effect of calcium hydroxide on bacterial lipopolysaccharide. J Endod 1993;19(2):76-8.

30. Buck, R.A., et al., Detoxification of endotoxin by endodontic irrigants and calcium hydroxide. J Endod 2001; 27(5):325-7.

31. Yassen, G.H., et al., Effect of medicaments used in endodontic regeneration technique on the chemical structure of human immature radicular dentin: an in vitro study. J Endod 2013; 39(2):269-73.

32. Sato, T., et al., In vitro antimicrobial susceptibility to combinations of drugs on bacteria from carious and endodontic lesions of human deciduous teeth. Oral Microbiol Immunol 1993;8(3):172-6.

33. Hoshino, E., et al., In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J 1996;29(2):125-30.

34. Petrino, J.A., et al., Challenges in regenerative endodontics: a case series. J Endod 2010; 36(3):536-41.

35. Kim, J.H., et al., Tooth discoloration of immature permanent incisor associated with triple antibiotic therapy: a case report. J Endod 2010;36(6):1086-91.

36. Iwaya, S.I., M. Ikawa, and M. Kubota, Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. Dent Traumatol 2001; 17(4):185-7.

37. Ruparel, N.B., et al., Direct effect of intracanal medicaments on survival of stem cells of the apical papilla. J Endod 2012;38(10):1372-5.

38. Nevins, A.J. and J.J. Cymerman, Revitalization of open apex teeth with apical periodontitis using a collagen-hydroxyapatite scaffold. J Endod 2015; 41(6):966-73.

39. Sabrah, A.H., G.H. Yassen, and R.L. Gregory, Effectiveness of antibiotic medicaments against biofilm formation of Enterococcus faecalis and Porphyromonas gingivalis. J Endod 2013; 39(11):1385-9.

40. Sabrah, A.H., et al., The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established Enterococcus faecalis biofilm. Clin Oral Investig 2015;19(8):2059-66.

41. Kim, K.W., et al., The effects of radicular dentine treated with double antibiotic paste and ethylenediaminetetraacetic acid on the attachment and proliferation of dental pulp stem cells. Dent Traumatol 2015;31(5):374-9.

42. Tzanetakis G.N., et al. Comparison of bacterial community composition of primary and persistent endodontic infections using pyrosequencing. J Endod 2015;41(8):1226-33.

43. Siqueira J.F., et al.Checkerboard DNA-DNA hybridization analysis of endodontic infections. Oral Surg, Oral Med, Oral Pathol, Oral Radiol, and Endod 2000;89(6):744-8.

44. Sassone L., et al. Evaluation of the microbiota of primary endodontic infections using checkerboard DNA–DNA hybridization. Oral Microbiol Immunol 2007; 22(6):390-7.

45. Basrani, B., A. Ghanem, and L. Tjaderhane, Physical and chemical properties of chlorhexidine and calcium hydroxide-containing medications. J Endod 2004;30(6):413-7.

46. Alacam T. G. Gorgul, and H. Omurlu, Evaluation of diagnostic radiopaque contrast materials used with calcium hydroxide. J Endod 1990;16(8):365-8.

47. Gerabek, E.W., The tooth-worm: historical aspects of a popular medical belief. Clin Oral Investig; 3(1):1-6.

48. Fitzharris, L. The Battle of the tooth worm. 2014 [cited 2018 January 4]; Available from: http://www.drlindseyfitzharris.com/2014/01/06/the-battle-of-the-tooth-worm/.

49. Cruse, W.P. and R. Bellizzi, A historic review of endodontics, 1689-1963 (Pt 1). J Endod 1980;6(3):495-9.

50. Grossman, L., A brief history of endodontics. J Endod 1982; 8:S36-S40.

51. Cruse, W.P. and R. Bellizzi, A historic review of endodontics, 1689-1963 (Pt 2) J Endod 1980; 6(4):532-5.

2. Curson, I., History and endodontics. Dent Pract Dent Rec 1965;15:435-9.

53. Koch, C.R.E. and B.L. Thorpe/ History of dental surgery.Chicago:The National Art Publishing Co.

54. Farley, J.R. Brief history of endodontics. Tex Dent J 1974;92(2):9.

55. Denton, G., The history of vitalism in pulp treatment. Dent Cosmos 1931;73: 267-73.

56. Anthony, L. and L. Grossman, A brief history of root canal therapy in the United States. J Am Dent Assoc 1945;32:43-50.

57. Grossman, L.I., Endodontics: a peep into the past and the future. Oral Surg Oral Med Oral Pathol 1974;37(4):599-608.

58. Coolidge, E.D., Past and present concepts in endodontics. J Am Dent Assoc 1960; 61: 676-88.

59. Francke, O.C., Capping of the living pulp: from Philip Pfaff to John Wessler. Bull Hist Dent 1971;19(2):17-23.

60. Ostrander, F.D., The practice of endodontics: past, present, and future. J Dent Educ 1967;31(3):386-8.

61. Jacobsohn, P.H. and R.J. Fedran, Making darkness visible: the discovery of X-ray and its introduction to dentistry. J Am Dent Assoc 1995;126(10):1359-67.

62. Pallasch, T.J. and M.J. Wahl, The focal infection theory: appraisal and reappraisal. J Calif Dent Assoc 2000;28(3):194-200.

63. Cruse, W.P. and R. Bellizzi, A historic review of endodontics, 1689-1963 (Pt 3). J Endod 1980; 6(5):576-80.

64. Hunter, W., The role of sepsis and of antisepsis in medicine. Dent Cosmos 1918: 585-602.

65. Glick, D.H., Endodontics: past, present and future. Alpha Omegan 1968; 61(2):. 124-6.

66. Grossman, L.I., Endodontics 1776-1976: a bicentennial history against the background of general dentistry. J Am Dent Assoc 1976;93(1):78-87.

67. Karapinar-Kazandag, M., B.R. Basrani, and S. Friedman, The operating microscope enhances detection and negotiation of accessory mesial canals in mandibular molars. J Endod 2010;36(8): 1289-94.

68. Kim, S. and S. Kratchman, Modern endodontic surgery concepts and practice: a review. J Endod 2006. 32(7):601-23.

69. Torabinejad, M., et al., Physical and chemical properties of a new root-end filling material. J Endod 1995; 21(7):349-53.

70. Patel, S., et al., The detection and management of root resorption lesions using intraoral radiography and cone beam computed tomography - an in vivo investigation. Int Endod J 2009;42(9):831-8.

71. Abella, F., et al., Evaluating the periapical status of teeth with irreversible pulpitis by using cone-beam computed tomography scanning and periapical radiographs. J Endod 2012; 38(12):1588-91.

72. Setzer, F.C., et al., Outcome of endodontic surgery: a meta-analysis of the literature--part 1: Comparison of traditional root-end surgery and endodontic microsurgery. J Endod 2010; 36(11):1757-65.

73. Gutmann, J., AAE history: 1990-2011.
.

74. Kakehashi, S., H.R. Stanley, and R.J. Fitzgerald, The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol 1965;20:340-9.

75. Peters, O.A. and C.I. Peters, Cleaning and shaping of the root canal system.In: Pathways of the pulp, Cohen S, H.K., eds. St Louis: Mosby Co., 2006, p.181-201.

76. Johnson, W. and W. Noblett, Cleaning and shaping, in Endodontics: principles and practice, M. Torabinejad and R. Walton, Editors. 2009, Saunders: St. Louis. p. 258-86.

77. Seltzer, S., et al., Pulpitis-induced interradicular periodontal changes in experimental animals. J Periodontol 1967;38(2):124-9.

78. Hulsmann, M., C. Rummelin, and F. Schafers, Root canal cleanliness after preparation with different endodontic handpieces and hand instruments: a comparative SEM investigation. J Endod 1997; 23(5):301-6.

79. Abbott, P.V., The periapical space--a dynamic interface. Aust Endod J 2002 28(3): 96-107.

80. Stewart, G.G., The importance of chemomechanical preparation of the root canal. Oral Surg Oral Med Oral Pathol 1955;8(9):993-7.

81. Grossman, L.I., Rationale of endodontic treatment. Dent Clin North Am 1967: 483-90.

82. Schilder, H., Filling root canals in three dimensions. Dent Clin North Am 1967; 723-44.

83. Ford, T. Relation between seal of root fillings and tissue response. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1983;55:291-4.

84. Heuer, M.A., [Endodontic Therapy (Biomechanic Preparation)]. Dent Cadmos 1965;33:17-8 PASSIM.

85. Khademi, A., M. Yazdizadeh, and M. Feizianfard, Determination of the minimum instrumentation size for penetration of irrigants to the apical third of root canal systems. J Endod 2006;32(5):417-20.

86. Pettiette, M.T., E.O. Delano, and M. Trope, Evaluation of success rate of endodontic treatment performed by students with stainless-steel K-files and nickel-titanium hand files. J Endod 2001; 27(2):124-7.

87. Peters, O.A., K. Schonenberger, and A. Laib, Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J 2001;34(3):221-30.

88. Peters, O.A., et al., Changes in root canal geometry after preparation assessed by high-resolution computed tomography. J Endod 2001;27(1): p. 1-6.

89. Hubscher, W., F. Barbakow, and O.A. Peters, Root-canal preparation with FlexMaster: canal shapes analysed by micro-computed tomography. Int Endod J 2003 36(11):740-7.

90. De Deus, Q.D., Frequency, location, and direction of the lateral, secondary, and accessory canals. J Endod 1975;1(11):361-6.

91. Love, R.M. and H.F. Jenkinson, Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 2002;13(2):171-83.

92. McDonnell, G. and A.D. Russell, Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 1999;12(1):147-79.

93. Barrette, W.C., Jr., et al., General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. Biochemistry 1989;28(23): 9172-8.

94. McKenna, S.M. and K.J. Davies, The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. Biochem J 1988;254(3):685-92.

95. Calt, S. and A. Serper, Time-dependent effects of EDTA on dentin structures. J Endod 2002;28(1): 17-9.

96. Niu, W., et al., A scanning electron microscopic study of dentinal erosion by final irrigation with EDTA and NaOCl solutions. Int Endod J 2002;35(11):934-9.

97. Shahravan, A., et al., Effect of smear layer on sealing ability of canal obturation: a systematic review and meta-analysis. J Endod 2007; 33(2):96-105.

98. Martinho, F.C. and B.P. Gomes, Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite. J Endod 2008; 34(3):268-72.

99. Weber, C.D., et al., The effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% sodium hypochlorite irrigant on residual antimicrobial activity in root canals. J Endod 2003;29(9):562-4.

100. Baca, P., et al., Residual effectiveness of final irrigation regimens on Enteroccus faecalis-infected root canals. J Endod 2011; 37(8):1121-3.

101. Hennessey, T.S., Some antibacterial properties of chlorhexidine. J Periodontal Res Suppl 1973;12:61-7.

102. Hugo, W.B. and A.R. Longworth, The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of Escherichia coli and Staphylococcus aureus. J Pharm Pharmacol 1966;18(9): 569-78.

103. Basrani, B.R., et al., Determination of 4-chloroaniline and its derivatives formed in the interaction of sodium hypochlorite and chlorhexidine by using gas chromatography. J Endod 2010; 36(2):312-4.

104. Nowicki, J.B. and D.S. Sem, An in vitro spectroscopic analysis to determine the chemical composition of the precipitate formed by mixing sodium hypochlorite and chlorhexidine. J Endod 2011;37(7):983-8.

105. Schaeffer, M.A., R.R. White, and R.E. Walton, Determining the optimal obturation length: a meta-analysis of literature. J Endod 2005; 31(4):271-4.

106. Salehrabi, R. and I. Rotstein, Endodontic treatment outcomes in a large patient population in the USA: an epidemiological study. J Endod 2004;30(12):846-50.

107. Hargreaves, K.M., S. Cohen, and L.H. Berman, Cohen's pathways of the pulp. 10th ed. St. Louis: Mosby Elsevier, 2011, p. xvi, 952, I34.

108. Cvek, M., A clinical report on partial pulpotomy and capping with calcium hydroxide in permanent incisors with complicated crown fracture. J Endod 1978; 4(8): 232-7.

109. Asgary, S., et al. A comparative study of histologic response to different pulp capping materials and a novel endodontic cement. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106(4): 609-14.

110. Lenherr, P., et al., Tooth discoloration induced by endodontic materials: a laboratory study. Int Endod J 2012;45(10):942-9.

111. Ham, J.W., S.S. Patterson, and D.F. Mitchell, Induced apical closure of immature pulpless teeth in monkeys. Oral Surg Oral Med Oral Pathol 1972;33(3):438-49.

112. Yassen, G.H. and J.A. Platt, The effect of nonsetting calcium hydroxide on root fracture and mechanical properties of radicular dentine: a systematic review. Int Endod J 2012.

113. Rosenberg, B., P.E. Murray, and K. Namerow, The effect of calcium hydroxide root filling on dentin fracture strength. Dent Traumatol 2007;23(1):26-9.

114. Doyon, G.E., T. Dumsha, and J.A. von Fraunhofer, Fracture resistance of human root dentin exposed to intracanal calcium hydroxide. J Endod 2005;31(12):895-7.

115. Valois, C.R. and E.D. Costa, Jr., Influence of the thickness of mineral trioxide aggregate on sealing ability of root-end fillings in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 97(1):108-11.

116. Witherspoon, D.E. and K. Ham, One-visit apexification: technique for inducing root-end barrier formation in apical closures. Pract Proced Aesthet Dent 2001; 13(6):455-60; quiz 462.

117. Holden, D.T., et al., Clinical outcomes of artificial root-end barriers with mineral trioxide aggregate in teeth with immature apices. J Endod 2008;34(7):812-7.

118. Ostby, B.N., The role of the blood clot in endodontic therapy. An experimental histologic study. Acta Odontol Scand 1961;19:324-53.

119. Rule, D.C. and G.B. Winter, Root growth and apical repair subsequent to pulpal necrosis in children. Br Dent J 1966;120(12):586-90.

120. Hargreaves, K.M., et al., Regeneration potential of the young permanent tooth: what does the future hold? J Endod 2008;34(7 Suppl):S51-6.

121. Murray, P.E., F. Garcia-Godoy, and K.M. Hargreaves, Regenerative endodontics: a review of current status and a call for action. J Endod 2007; 33(4):377-90.

122. Huang, G.T., Dental pulp and dentin tissue engineering and regeneration: advancement and challenge. Front Biosci (Elite Ed) 2011;3:788-800.

123. Fouad, A.F., The microbial challenge to pulp regeneration. Adv Dent Res 2011; 23(3):285-9.

124. Nakashima, M. Tissue engineering in endodontics. Aust Endod J 2005;31(3): 111-3.

125. Sedgley, C.M. and T.M. Botero, Dental stem cells and their sources. Dent Clin North Am 2012;56(3):549-61.

126. Saito, D., et al., Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries. J Med Microbiol 2006;55(Pt 1):101-7.

127. Nagata, J.Y., et al., Microbial evaluation of traumatized teeth treated with triple antibiotic paste or calcium hydroxide with 2% chlorhexidine gel in pulp revascularization. J Endod 2014; 40(6):778-83.

128. Nair, P.N., et al., Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. J Endod 1990;16(12):580-8.

129. Siqueira, J., Treatment of endodontic infections. London: Quintessence, 2007.

130. Sato, I., et al., Sterilization of infected root-canal dentine by topical application of a mixture of ciprofloxacin, metronidazole and minocycline in situ. Int Endod 1996; 29(2):118-24.

131. Labban, N., et al. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. Dent Traumatol 2014.

132. Althumairy, R.I., F.B. Teixeira, and A. Diogenes, Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. J Endod 2014; 40(4):521-5.

133. Yassen, G.H., G.J. Eckert, and J.A. Platt, Effect of intracanal medicaments used in endodontic regeneration procedures on microhardness and chemical structure of dentin. Restor Dent Endod 2015;40(2):104-12.

134. McTigue, D.J., K. Subramanian, and A. Kumar, Case series: management of immature permanent teeth with pulpal necrosis: a case series. Pediatr Dent 2013; 35(1):55-60.

135. Gomes, B.P., et al., Analysis of the antimicrobial susceptibility of anaerobic bacteria isolated from endodontic infections in Brazil during a period of nine years. J Endod 2011;37(8):1058-62.

136. Skucaite, N., et al., Susceptibility of endodontic pathogens to antibiotics in patients with symptomatic apical periodontitis. J Endod 2010;36(10):1611-6.

137. Akcay, M., et al., Spectrophotometric analysis of crown discoloration induced by various antibiotic pastes used in revascularization. J Endod 2014;40(6): 845-8.

138. Tagelsir, A., et al., Effect of antimicrobials used in regenerative endodontic procedures on 3-week-old Enterococcus faecalis biofilm. J Endod 2016;42(2):. 258-62.

139. Berman, L.H., ed. Cohen's pathways of the pulp.10th ed. St. Louis: Mosby Elsevier, 2011:1079.

140. Siqueira, J.F., Jr. and H.P. Lopes, Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999;32(5):361-9.

141. Yassen, G.H., et al., The effect of medicaments used in endodontic regeneration on root fracture and microhardness of radicular dentine. Int Endod J 2013;46(7): 688-95.

142. Senia, E.S., F.J. Marshall, and S. Rosen, The solvent action of sodium hypochlorite on pulp tissue of extracted teeth. Oral Surg Oral Med Oral Pathol, 1971. 31(1): p. 96-103.

143. Siqueira, J.F., Jr., et al., Efficacy of instrumentation techniques and irrigation regimens in reducing the bacterial population within root canals. J Endod 2002; 28(3):181-4.

144. Vianna, M.E., et al., In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97(1):79-84.

145. Yesilsoy, C., et al., Antimicrobial and toxic effects of established and potential root canal irrigants. J Endod 1995;21(10):513-5.

146. Sirtes, G., et al., The effects of temperature on sodium hypochlorite short-term stability, pulp dissolution capacity, and antimicrobial efficacy. J Endod 2005;. 31(9): 669-71.

147. Baumgartner, J.C. and P.R. Cuenin, Efficacy of several concentrations of sodium hypochlorite for root canal irrigation. J Endod 1992;18(12): 605-12

.

148. Martin DE, H.M., Almeida JFA, Teixeira FB, Hargreaves KM, Diogenes AR., Effect of sodium hypochlorite on the odontoblastic phenotype differentiation of SCAP in cultured organotype human roots. J Endod 2012(Mar):e26.

149. Pang, N.S., et al., Effect of EDTA on attachment and differentiation of dental pulp stem cells. J Endod, 2014. 40(6): p. 811-7.

150. American Association of Endodontists. AAE Clinical Considerations for a Regenerative Procedure. Available at:https://www.aae.org/specialty/wp-content/uploads/sites/2/2017/06/currentregenerativeendodonticconsiderations.pdf Accessed January 3, 2018. 2018.

151. Torabinejad, M., et al., Clinical implications of the smear layer in endodontics: a review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002; 94(6):658-66.

152. Trevino, E.G., et al., Effect of irrigants on the survival of human stem cells of the apical papilla in a platelet-rich plasma scaffold in human root tips. J Endod 2011. 37(8):1109-15.

153. Yamauchi, N., et al., Immunohistological characterization of newly formed tissues after regenerative procedure in immature dog teeth. J Endod 2011;37(12): 1636-41.

154. Gronthos, S., et al. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 2000;97(25):13625-30.

155. Seo, B.M., et al., Recovery of stem cells from cryopreserved periodontal ligament. J Dent Res 2005;84(10):907-12.

156. Miura, M., et al., SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 2003;100(10):5807-12.

157. Morsczeck, C., et al., Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol 2005;24(2):155-65.

158. Huang, G.T., et al., The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. J Endod 2008;34(6):645-51.

.

159. Sonoyama, W., et al., Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. J Endod 2008; 34(2): 166-71.

160. Sonoyama, W., et al. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One 2006;1:e79.

161. Nevins, A.J., et al., Revitalization of pulpless open apex teeth in rhesus monkeys, using collagen-calcium phosphate gel. J Endod 1976;2(6):159-65.

162. Thibodeau, B., et al., Pulp revascularization of immature dog teeth with apical periodontitis. J Endod 2007;33(6):680-9.

163. Hutmacher, D.W., Scaffold design and fabrication technologies for engineering tissues--state of the art and future perspectives. J Biomater Sci Polym Ed 2001; 12(1):107-24.

164. Torabinejad, M. and H. Faras, A clinical and histological report of a tooth with an open apex treated with regenerative endodontics using platelet-rich plasma. J Endod 2012;38(6): 864-8.

165. Torabinejad, M. and M. Turman, Revitalization of tooth with necrotic pulp and open apex by using platelet-rich plasma: a case report. J Endod 2011;37(2):265-8.

166. Smith, A.J., Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. J Dent Educ 2003 67(6):678-89.

167. Wei X, L.J., Wu L, Liu L, Xiao Y, Expression of mineralization markers in dental pulp cells. J Endod 2007; 33(6):703-8.

168. Okamoto Y, S.W., Ono M, Akiyama K, Fujisawa T, Oshima M, and M.Y. Tsuchimoto Y, Yasuda T, Shi S, Kuboki T, Simvastatin induces the odontogenic differentiation of human dental pulp stem cells in vitro and in vivo. J Endod 2009; 35(3):367-72.

169. Kahler, B. et al.Revascularization outcomes: a prospective analysis of 16 consecutive cases. J Endod 2014; 40(3):333-8.

170. Wigler R, K.A., Lin S, Steinbock N, Hazan-Molina H, Torneck CD, Revascularization: a treatment for permanent teeth with necrotic pulp and incomplete root development. J Endod 2013; 39(3):319-26.

171. Du, T., et al., Effect of long-term exposure to endodontic disinfecting solutions on young and old Enterococcus faecalis biofilms in dentin canals. J Endod 2014;. 40(4):509-14.

172. Wang, Z., Y. Shen, and M. Haapasalo, Effectiveness of endodontic disinfecting solutions against young and old Enterococcus faecalis biofilms in dentin canals. J Endod 2012;38(10):1376-9.

173. Prather, B.T., et al., Effects of two combinations of triple antibiotic paste used in endodontic regeneration on root microhardness and chemical structure of radicular dentine. J Oral Sci 2014;56(4):245-51.

174. Sassone, L.M., et al., A microbiological profile of unexposed and exposed pulp space of primary endodontic infections by checkerboard DNA-DNA hybridization. J Endod 2012;38(7):889-93.

175. Takushige T, C.E., Asgor Moral A, Hoshino E, Endodontic treatment of primary teeth using a combination of antibacterial drugs. Int Endod J 2004;37:132-8.

176. Jacobs JC, T.A., Ehrlich Y, Spolnik K, Bringas JS, Gregory RL, Yassen GH, Antibacterial effects of antimicrobials used in regenerative endodontics againist biofilm bacteria from mature and immature teeth with necrotic pulps. J Endod 2017;43(4):575–9.

177. Ordinola-Zapata, R., et al., The effect of radiopacifiers agents on pH, calcium release, radiopacity, and antimicrobial properties of different calcium hydroxide dressings. Microsc Res Tech 2015;78(7):620-5.

178. Hungaro Duarte, M.A., et al., Radiopacity of portland cement associated with different radiopacifying agents. J Endod 2009; 35(5):737-40.

179. Camilleri J, G.M., Evaluation of the radiopacity of calcium silicate cements containing different radiopacifiers. Int Endod J 2010;43:21–30.

180. Pascon, E.A. and L.S. Spangberg, In vitro cytotoxicity of root canal filling materials: (Pt 1) Gutta-percha. J Endod 1990;16(9): 429-33.

181. Ribeiro DA, C.V., Fracalossi ACC, Oyama LM, Radiopacifiers do not induce genetic damage in murine fibroblasts: an in vitro study. Int Endod J 2009;.42: 987–91.

182. Eftekhar B, M.E., Eini E, Jafarzadeh M, Behrooz N. Evaluation of hydroxyl ion diffusion in dentin and injectable forms and a simple powder-water calcium hydroxide paste: an in vitro study. Jundishapur J Natural Pharmaceut Product 2014; 9(3):e14029.

183. Blanscet ML, T.P., Goodell GG, An agar diffusion comparison of the antimicrobial effect of calcium hydroxide at five different concentrations with three different vehicles. J Endod 2008;34(10):1246–8.

184. Behnen MJ, W.L., Liewehr FR, Buxton TB, McPherson JC, Antimicrobial activity of several calcium hydroxide preparations in root canal dentin. J Endod 2001; 27(12):765-67.

185. Alghilan, M.A., et al., Attachment and proliferation of dental pulp stem cells on dentine treated with different regenerative endodontic protocols. Int Endod J 2016. Accepted for publication.

186. Jenks, D.B., et al., Residual antibiofilm effects of various concentrations of double antibiotic paste used during regenerative endodontics after different application times. Arch Oral Biol 2016;70:88-93.

187. Hsiao, W.W., et al., Microbial transformation from normal oral microbiota to acute endodontic infections. BMC Genomics 2012;13:345.

188. Wong KS, S.-L.V., The effect of intracanal Ledermix on root resorption of delayed-replanted monkey teeth. Dent Traumatol 2002;18:309-15.

189. Li, L., et al., Analyzing endodontic infections by deep coverage pyrosequencing. J Dent Res 2010;89(9):980-4.

ABSTRACT

THE ANTIBACTERIAL EFFECTS OF RADIOPAQUE DOUBLE ANTIBIOTIC PASTES AGAINST CLINICAL BACTERIAL ISOLATES FROM MATURE AND IMMATURE TEETH WITH NECROTIC PULPS

by

Carolin Francis Ibrahim

Indiana University School of Dentistry
Indianapolis, Indiana

Low concentrations (1 mg/mL to 10 mg/mL) of double antibiotic paste (DAP) have demonstrated antibacterial properties in regenerative endodontics. The aim of this study was to evaluate if DAP made radiopaque (RoDAP) with barium sulfate has antibacterial effects against bacterial isolates from a mature and immature tooth with necrotic pulp.

Clinical bacterial isolates were obtained from the canals of mature and immature teeth with necrotic pulps during root canal therapy or a regenerative procedure, respectively. Bacterial isolates were grown anaerobically for three weeks on 4x4x1-mm dentin specimens prepared from extracted human teeth (n = 48 per biofilm type). The dentin specimens were allocated into six groups and treated as follows: 1mg/mL RoDAP, 10 mg/mL RoDAP, calcium hydroxide (UltraCal XS), placebo (barium sulfate in methylcellulose), no treatment, and no bacteria or treatment (sterile control). After one week of treatment the biofilm was detached and biofilm disruption assays were conducted to determine the bacterial numbers (CFUs/mL). The data were analyzed using

Wilcoxon Rank Sum tests followed by pairwise comparisons. Treatments with 1 mg/ML and 10 mg/mL RoDAP as well as UltraCal XS demonstrated significant antibacterial effects against the tested bacterial isolates. The placebo paste did not demonstrate any significant antibacterial effects. No significant difference in antibacterial effects was found against isolates from both mature and immature teeth regardless of the type of treatment.

Both 1 mg/mL and 10 mg/mL RoDAP demonstrated significant antibacterial effects against bacterial isolates from mature and immature teeth with necrotic pulps. RoDAP may be beneficial in clinical applications since its adequate placement within the canal system can be confirmed radiographically.

CURRICULUM VITAE

Carolin Francis Ibrahim

March 24th 1987	Born to Bernadet Yacoub & Francis Rayis Baghdad, Iraq
2005 to 2009	Bachelor of Science (BS), Biology Oakland University Rochester, MI
2009 to 2013	Doctor of Dental Surgery (DDS) University of Detroit Mercy School of Dentistry Detroit, MI
July 2013 to May 2014	General Dentist Bright Side Dental Clarkston, MI and Royal Oak, MI
Oct 2013 to Sept 2014	General Dentist Marine City Dental Marine City, MI
Sept 2014 to May 2016	General Dentist Southfield Family Dental Center Southfield, MI
2016 to 2018	Endodontic resident and graduate student Indiana University School of Dentistry Indianapolis, IN
June 30, 2018	MSD and Certificate in Endodontics Indiana University School of Dentistry Indianapolis, IN

Professional Associations

American Association of Endodontists (AAE)
American Dental Association (ADA)
Michigan Dental Association (MDA)